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Age-Related Decrease in Hypothalmic Gonadotropin-Releasing Hormone (GnRH) Gene Expression, But Not Pituitary Responsiveness to GnRH, in the Male Brown Norway Rat

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ABSTRACT: As is the case in humans, aging male Brown Norway (BN) rats exhibit both primary and secondary (hypothalamic/pituitary) testicular failure. We hypothesized that secondary testicular failure in aging BN rats is due to alterations in both hypothalamic and pituitary function. In order to determine whether gonadotropin-releasing hormone (GnRH) gene expression is altered with aging, we compared hypothalamic preproGnRH (ppGnRH) mRNA by in situ hybridization histochemistry and GnRH peptide content in microdissected brain areas by radioimmunoassay in intact (or sham-operated) young, middle-aged, and old male rats. In addition, we determined hypothalamic-pituitary responsiveness to the removal of testicular feedback by comparing ppGnRH messenger RNA (mRNA) and gonadotropin levels in sham-operated and orchidectomized young, middle-aged, and old rats. In sham-operated rats, both the cellular ppGnRH mRNA content and the number of neurons expressing ppGnRH mRNA were lower in old compared with young and middle-aged rats. In addition, GnRH content decreased with aging in intact rats in 2 of the 3 brain areas examined, and GnRH content tended to decrease with aging in the third region. Morning serum luteinizing hormone (LH) levels were unchanged with aging, whereas follicle-stimulating hormone (FSH) was significantly increased in old compared with younger intact rats. The cellular ppGnRH mRNA content also decreased with aging in orchidectomized rats, although the number of neurons expressing ppGnRH mRNA was unchanged with aging in these rats. Within age groups, the cellular ppGnRH mRNA content was higher in orchidectomized than in sham-operated rats, though there was no effect on the number of neurons expressing GnRH. In a second study, we compared pituitary responsiveness to GnRH by measuring serum LH and FSH levels after GnRH administration in intact BN rats of different ages. The LH response to GnRH was unchanged with aging, whereas the FSH response to GnRH tended to increase with aging. Despite similar LH responses, the testosterone (T) response to GnRH declined progressively with aging. A third study assessed age-related changes in the circadian rhythm of circulating LH, T, and corticosterone (B) levels. LH levels over a 24-hour period decreased with aging and tended to be lower in the morning hours in all age groups, and circadian rhythmicity was blunted in middle-aged and old compared with young rats. T levels over 24 hours declined progressively with aging, and these levels showed a bimodal diurnal variation in young rats, a variation that was not evident in older animals. B levels over a 24-hour period were lower in old than in younger animals, and with aging, there was dampening of the amplitude of the circadian rhythm of B. Taken together, these findings suggest that secondary testicular failure in aging male BN rats is due in part to decreased GnRH gene expression rather than to decreased pituitary responsiveness to GnRH. This reduction in GnRH gene expression with aging is not dependent on testicular feedback factors. Finally, the blunted circadian rhythmicity of LH and T secretion with aging provides further evidence of altered hypothalamic regulation of gonadal hormone secretion in old animals.

Key words: Messenger RNA, aging, reproduction, testosterone, in situ hybridization, circadian rhythms.

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In man, reproductive system aging is characterized by both primary testicular failure and secondary (hypothalamic/pituitary) testicular dysfunction. It is not possible to directly assess hypothalamic function in man, but work in animal models has provided insight into aging-related changes in hypothalamic function. In the male rat, age-associated reproductive decline is thought to be due in part to diminished gonadotropin-releasing hormone (GnRH) secretion. It has been reported that hypothalamic GnRH gene expression is decreased in aging male rat models, such as the Fischer 344 (F344) rat (Gruenewald and Matsumoto, 1991a), which suggests that a reduction in GnRH synthetic capacity may contribute to the age-associated decline in gonadotropin secretion and reproductive function. However, most male rat models of aging, including the F344, exhibit mainly secondary testicular failure, thereby reducing their usefulness as a model

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of human reproductive aging (Gruenewald et al, 1994a). In addition, the presence of functional progesterone-secreting testicular tumors and prolactin-secreting pituitary tumors in aging male rat models (such as the F344, BNxF344, and other strains) further confounds the results of studies that use these models (Amador et al, 1985; Bartke et al, 1985; Gruenewald et al, 1992, 1994a; Thurman et al, 1995). Indeed, it is possible that the reported decline in GnRH synthetic capacity and reproductive function in aging male F344 rats reflected a suppressive effect of excessive progesterone or prolactin secretion on the reproductive hypothalamic/pituitary axis rather than aging per se.

We (Gruenewald et al, 1994a) and others (Wang et al, 1993; Zirkin et al, 1993) have reported evidence of both primary and secondary testicular failure in the aging male Brown Norway (BN) rat model, which is similar to the human male. In addition, we demonstrated that these rats do not exhibit excessive steroid secretion from testicular tumors or hyperprolactinemia, which suggests that the BN rat is an excellent model for human male reproductive aging. In the present study, we hypothesized that secondary testicular failure in aging BN rats is due to alterations in both hypothalamic and pituitary function. In order to determine whether hypothalamic GnRH gene expression is altered with aging, we compared preproGnRH (ppGnRH) messenger RNA (mRNA) in the medial preoptic area (MPOA) and the diagonal band of Broca (DBB)—by in situ hybridization histochemistry—and GnRH peptide content in microdissected brain areas—by radioimmunoassay (RIA)—in intact young, middle-aged, and old male rats. In addition, we determined hypothalamic-pituitary responsiveness to removal of testicular feedback by comparing ppGnRH mRNA and gonadotropin levels in intact and orchidectomized young, middle-aged, and old rats. In order to determine whether pituitary responsiveness to GnRH is altered with aging, we measured serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone (T) levels after GnRH administration in intact BN rats of different ages. Finally, in order to evaluate the effects of aging on the circadian rhythms of LH and T, hormone levels were measured in rats at varying times throughout a 24-hour period.

Materials and Methods

Animals

Adult male BN rats that were 3 to 4 (young), 12 to 13 (middle aged), and 22 to 24 (old) months of age were purchased (National Institute on Aging colony, Harlan Sprague-Dawley, Indianapolis, Ind) and housed in a room containing only rats that were free of mycoplasma and detectable murine viruses. These ages were selected based on our earlier work in aging male BN rats, which found normal to increased gonadotropin levels and decreased testosterone and spermatogenesis similar to that of aging men in 23- to 24-month-old rats as well as a low probability that these changes are due to disease-related factors (Gruenewald et al, 1994a). (The reported 50% survival age for BN rats occurs at 28 to 30 months of age [Burek and Hollander, 1977].) Animals were maintained under the following light conditions: 12 hours light and 12 hours dark (lights were turned on at 0600 hours); animals were provided with ad libitum access to Purina rat chow and tap water. Animals were allowed a 14-day period of acclimatization prior to being used for these studies. All animal experimentation was conducted in accordance with principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Experimental Design

Study 1A—Three-, thirteen-, and twenty-three-month-old BN rats were randomly assigned to receive either bilateral orchidectomy (3 month, n = 8; 13 month, n = 7; 23 month, n = 7) or sham operation (3 month, n = 8; 13 month, n = 8; 23 month, n = 7) under methoxyflurane anesthesia. Animals were sacrificed 21 days after surgery. ppGnRH mRNA levels, quantified by in situ hybridization histochemistry (ISHH), were compared between orchidectomized and sham-operated animals and between age groups in anatomically matched sections of DBB and MPOA.

Study 1B—Intact 3-month-old (n = 12), 13-month-old (n = 12), and 22-month-old (n = 11) male BN rats were sacrificed after 14 days of acclimatization. GnRH peptide content, determined by RIA in microdissected punches from the MPOA, median eminence (ME), and arcuate nucleus (ARC), was compared between age groups. Serum T, LH, and FSH levels for rats in studies 1A and 1B were determined by RIA.

Study 2—After 14 days of acclimatization, 3-month-old (n = 9), 13-month-old, (n = 9), and 23- to 24-month-old (n = 10) male BN rats received an intravenous injection of GnRH (100 ng/100 g body weight) followed by serial blood sampling to determine LH and FSH responsiveness to GnRH administration.

Study 3—After 16 days of acclimatization, 3- to 4-month-old (n = 40), 12- to 13-month-old (n = 40), and 23- to 24-month-old (n = 46) male BN rats were sacrificed at varying times over a 24-hour period (n = 8 to 9 per time point) so that we could evaluate circadian variations in serum LH, T, and corticosterone (B) levels.

Study 1

Tissue Preparation and Blood Sample Collection—Rats were sacrificed by decapitation; brains were removed and quickly frozen on dry ice and were stored intact at −70°C until sectioning and processing for ISHH or peptide content determinations. Inspection of the pituitary fossa was performed in all animals, and rats with pituitary tumors were excluded (n = 5). Trunk blood was collected from all animals between 0800 and 1500 hours. Because animals were sacrificed over the course of this 7-hour period, an equal number of rats from each age group were sacrificed at intervals throughout this time period in order to minimize the potential confounding effects of circadian variations in

hormone levels. Serum was stored at −20°C until T, LH, and FSH levels were determined by RIA.

For ISHH (study 1A), RNase-free procedures were used during tissue sectioning, prehybridization, and hybridization. Serial 20-μm frozen coronal sections were obtained through the MPOA and DBB of each brain, and these sections were thaw-mounted onto gelatin-subbed RNase-free microscope slides. Slides were stored at −70°C until samples were hybridized.

For GnRH content determinations (study 1B), serial 300-μm sections were obtained through the MPOA, ME, and ARC of each brain. The ME was dissected using a fine scalpel, and the MPOA and ARC were microdissected using a 300-μm punch, according to the method of Palkovits and Brownstein (Palkovits and Brownstein, 1983). GnRH content was determined by RIA in 6 to 12 microdissected samples per rat from each nuclear region, using methods previously described (Matsumoto et al, 1986). Microdissected tissue samples were extracted in 250 μL of boiling 1 M acetic acid for 10 minutes. After cooling and sonication, 125 μL of the suspension was removed and lyophilized. The remaining suspension was used for protein determination using the method of Bradford (Bradford, 1976).

Probe (Study 1A)—A 48-base oligodeoxynucleotide probe complementary to the entire GnRH-encoding region and the first 18 bases of the GAP-encoding region of ppGnRH cDNA were synthesized by the Molecular Biology Core facility at the Veterans Affairs Puget Sound Health Care System, Seattle Division (Seattle, Wash). Probe was purified in our laboratory using polyacrylamide gel electrophoresis under denaturing conditions. Probe was 3′ end-labeled with [^{35}S]deoxyadenosine triphosphate (dATP), purified, and reconstituted in TED buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA, 10 mM dithiothreitol), as described elsewhere (Gruenewald and Matsumoto, 1991a). The hybridization and probe specificity of this probe have been documented previously and have been verified in our laboratory, as described previously (Gruenewald and Matsumoto, 1991a).

Hybridization Procedures (Study 1A)—In situ hybridization was performed as described previously (Gruenewald and Matsumoto, 1991a,b). Briefly, tissue sections were fixed in 4% paraformaldehyde (pH 7.4) for 5 minutes, washed in phosphate-buffered saline, rinsed in 0.1 M triethanolamine (pH 8.0), and treated with 0.25% acetic anhydride. The tissue was rinsed in 2× standard saline citrate (SSC), dehydrated in a series of ethanol solutions, delipidated in chloroform, passed through a second series of ethanol rinses, and air-dried.

Labeled probe mixture (including yeast tRNA and TED buffer) was diluted in hybridization buffer to a saturating concentration of 1.2 μg/[mL × kilobase]. Aliquots of this hybridization mixture were applied to each slide, coverslips were applied, and slides were incubated overnight at 37°C in moist chambers. Coverslips were removed in 1× SSC, and slides were passed through successive 1× SSC washes at 62°C, followed by 2 1× SSC washes at room temperature. Tissue sections were dehydrated through a series of ethanol solutions that contained ammonium acetate and were then air-dried.

Autoradiography and Histological Staining (Study 1A)—Slides were dipped in warm Kodak NTB2 emulsion (Eastman Kodak, Rochester, NY), air-dried, and stored at 4°C. After a 15-day exposure period, slides were developed in Kodak D-19, counterstained with cresyl violet, and coverslipped.

Anatomical Matching and Quantitation of ppGnRH mRNA (Study 1A)—Using the rat brain atlas of Konig and Klippel (1963), anatomically matched coronal sections from each animal were identified for analysis by ISHH. Four sections per animal were matched and analyzed from the DBB, 6 sections per animal were matched and analyzed from the rostral MPOA, 4 sections per animal were matched and analyzed from the middle MPOA, and 4 sections per animal were matched and analyzed from the caudal MPOA (atlas plates 16 to 19 [A 7890 to 7020 μ] [Konig and Klippel, 1963]), for a total of 18 sections per rat.

Slides were coded and analyzed in random order, without knowledge of the animal from which the tissue was derived. Cellular ppGnRH mRNA content was quantified using a BioScan Optimas computerized image-analysis system (Edmonds, Wash), as described elsewhere (Gruenewald and Matsumoto, 1991a). Cells expressing the GnRH gene were identified under dark-field microscopy by the localization of silver grain clusters overlying cell nuclei, as follows: a gray level value was assigned to each pixel within a digitized image of a field of interest. Silver grain clusters identifying labeled cell nuclei were then identified by manually setting an optimal threshold value for each field that best distinguished the labeled cell nuclei from the background. The presence of cresyl violet–stained cell nuclei was confirmed using light-field illumination, and grain clusters not overlying cell nuclei were deselected. The number of labeled nuclei within the field was then counted under dark-field imaging by the imaging system. For each silver grain cluster, the number of silver grains per cell was quantified by the BioScan Optimas computerized image-analysis system utilizing a grain-detection program adapted from a previously used program, as described in detail elsewhere (Gruenewald and Matsumoto, 1991b). The number of silver grains per cell was used as an index of cellular ppGnRH mRNA content, and the number of labeled cells counted per brain section was used as an index of the number of neurons expressing the GnRH gene.

Study 2

Prior to GnRH administration and blood sample collection, animals were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg). After induction of anesthesia, an external jugular cutdown was performed, and a catheter was inserted into the external jugular vein for serial blood sampling. Blood (0.4 mL) was obtained for baseline gonadotropin determinations; extraction was followed by replacement of blood volume with an equal volume of isotonic saline solution. After completion of baseline blood sampling, the animals received GnRH (100 ng/100 g body weight). After intravenous GnRH was given, 0.4 mL of blood was removed every 10 minutes for 60 minutes, which was again followed by replacement of blood volume with an equal volume of isotonic saline solution. At 70 minutes after GnRH injection, animals were decapitated, and trunk blood was saved for gonadotropin and T levels. All blood samples were placed on ice and were subsequently frozen at −20°C until LH, FSH, and T measurements were performed by RIA.

Study 3

Rats of each age group (3 to 4 month, n = 40; 12 to 13 month, n = 40; 23 to 24 month, n = 46) were assigned randomly to one of 5 groups (n = 8 to 9 per group) to be sacrificed at different times over a 24-hour period (group 1 sacrificed at 2300 hours; group 2 at 0500 hours; group 3 at 1100 hours; group 4 at 1700 hours; and group 5 at 2300 hours). Throughout the course of the study, animals were maintained under the following light conditions: 12 hours light and 12 hours dark (lights were turned on at 0600 hours); beginning on day 16 (after rats were received from the National Institute on Aging colony), a darkroom red light was kept on at all times. Between days 26 and 29, groups of rats were sacrificed at their respective times, and trunk blood was collected for serum LH, FSH, and T measurements, as described above for study 1. Serum B levels were also measured as a control to confirm that we were able to detect circadian rhythmicity for a hormone with known circadian variations and in order to verify that the animals were not stressed (eg, Atkinson and Waddell, 1997). The times for sacrifice were determined as follows: one half of the rats from group 1 were sacrificed on day 26 and the remainder on day 28. For groups 2 through 5, one half of the rats from each group were sacrificed on day 27 and the rest on day 29. In this way, an equal number of rats was sacrificed from each age cohort in order to minimize any effect of sacrificing rats on different days, effects that might confound the effects of aging on hormone levels.

Radioimmunoassays

GnRH—GnRH content in lyophilized extracts from microdissected brain tissue was measured by a double-antibody RIA using methods described previously (Matsumoto et al, 1986). Polyclonal rabbit GnRH antiserum, N540, supplied by Dr Robert A Steiner (Department of Obstetrics and Gynecology, University of Washington) was used. The tracer used was ^{125}I-GnRH obtained from New England Nuclear (Boston, Mass). The assay sensitivity was 0.8 ng/tube, and the intra-assay coefficient of variation was 2%. Specificity of the N540 antiserum for the full mammalian GnRH decapeptide sequence has been demonstrated previously (Dorsa et al, 1984).

Gonadotropins—Serum LH and FSH levels were determined by RIA using reagents provided by the National Hormone and Pituitary Program of the NIDDK (directed by Dr Salvatore Raiti, University of Maryland School of Medicine, Baltimore, Md) and using methods described previously (Matsumoto et al, 1986). The antisera used were rabbit anti-rat LH S-7 and FSH S-11; the tracers used were rat LH I-7 and FSH I-7, radioiodinated with ^{125}I using chloramine-T. The reference standard used for both assays was RP-2. For LH, the intra-assay and interassay coefficients of variation were 2.9 and 11%, respectively, and the assay sensitivity was 0.06 ng/mL, except for in study 1A, where the sensitivity limit was 0.14 ng/mL. LH levels were above the limit of detectability in all cases, except in study 1A, where 3 young (3-month-old), 2 middle-aged (13-month-old), and 4 old (23-month-old) rats had LH levels that were at or below 0.14 ng/mL. Because only a few rats with undetectable LH levels were distributed throughout all 3 age groups and because a much larger number of rats in study 1B all had detectable LH levels, we included these animals in the mean LH levels reported for the combined studies 1A and 1B. As a further check on the validity of the LH results, these samples were also run in the Delfia assay, which had a sensitivity of 0.03 ng/mL, which was well below observed values for all rats. The results that we found using the Delfia assay for studies 1A, 1B, and 3 were similar to those found with the original RIA, which supports the validity of the original data. Insufficient sample was available to rerun the study 2 samples with the Delfia assay, but in this study, even the baseline LH levels were above the detectability limit of the LH RIA. For FSH, intra- and interassay variabilities were 7.2 and 12.4%, respectively, and the sensitivity was 100 pg/tube.

Testosterone—Serum T levels were measured by RIA using reagents provided by the World Health Organization Matched Reagent Programme and using methods previously described (Tenover et al, 1987). T antiserum was raised in rabbits against T-bovine serum albumin, and it exhibited cross-reactivity of 14% with 5α-dihydrotestosterone, of 6% with 5α-androstanediol, and of less than 2% with the other steroids tested. The assay sensitivity was 0.35 nmol/L (0.1 ng/mL), and the intra- and interassay variabilities were 5.1 and 9.8%, respectively.

Corticosterone—Serum B levels were determined by RIA. Samples were assayed in duplicate in an initial volume of 0.5 mL of phosphate buffer containing 1 μL of serum. No extraction procedure was performed, but standards and samples were heated at 80°C for 20 minutes to denature B-binding globulin and other binding globulins. After addition of antiserum (stock #07-120016, ICN Pharmaceuticals, Costa Mesa, Calif) and (^{3}H)-B and following incubation for 16 hours at 4°C, cold dextran-coated charcoal was added, and the samples were centrifuged. The supernatant was then decanted into vials, scintillation fluid was added, and samples were counted. The antiserum exhibited cross-reactivity of 33% with progesterone, of 6.4% with 20α-dihydroprogesterone, of 5.3% with cortisol, of 2.1% with androstenedione, and of less than 2% with other steroids tested. Intra- and interassay coefficients of variation were 8.9 and 13.4%, respectively. Sensitivity of the assay was 5 pg/tube.

Statistical Analysis

In study 1A, the mean number of grains per cell and of labeled cells per section in the MPOA and DBB were determined for each brain section with detectable signal. Differences in grains/cell and cells/section between age groups and between orchidectomized and sham-operated animals were assessed by 2-way analysis of variance (ANOVA) with Scheffe's *F* test for multiple comparisons.

In study 1B, differences in GnRH content between young, middle-aged, and old rats were determined for each brain region by 1-way ANOVA with Scheffe's *F* test for multiple comparisons.

Serum T, LH, and FSH levels were determined by RIA in samples from sham-operated rats in study 1A and from intact rats in study 1B. Hormone levels were determined in different assays for the 2 studies, but the same RIA methods were used; therefore, the hormone data were combined and analyzed together. Differences in serum T, LH, and FSH levels between young, middle-aged, and old rats were determined by 1-way ANOVA with Scheffe's *F* test for multiple comparisons.

In study 2, responsiveness to GnRH stimulation was deter-

mined in 2 ways. First, the absolute changes in LH, FSH, and T from baseline to peak were determined for each animal. Second, the areas under the gonadotropin- and T-response curves (AUCs) were calculated for each animal by the trapezoidal rule (Dixon et al, 1990) and were adjusted for the baseline gonadotropin level. The mean differences in gonadotropin levels from baseline to peak and AUC measurements were then compared between age and treatment groups using 2-way ANOVA with Scheffe's *F* test for multiple comparisons.

Because of the small volume of blood sample available for analysis in study 2 for the measurements obtained at 10-minute intervals, inadequate samples were available for FSH and T determinations at some time points. Accordingly, FSH and T aliquots for adjacent time points were pooled, and the data were expressed for a time point that was intermediate between the adjacent time points, which reflected the average time of collection for all of the pooled samples.

For study 3, the area under the curve (AUC) over a 24-hour period was calculated for LH, T, and B levels by the trapezoidal rule (Dixon et al, 1990), and this area was compared between age groups. FSH data were not analyzed because an insufficient volume of the blood sample was available and because of problems with the FSH assay for this study. Because hormone levels were obtained infrequently (every 6 hours) over a 24-hour period and because they were obtained in different animals, no formal analysis of pulsatile hormone release (such as cosinor analysis) was undertaken. Instead, the absolute change from baseline to peak LH, T, and B levels over a 24-hour period was determined for each animal, and the mean differences were compared between age groups using 2-way ANOVA with Scheffe's *F* test for multiple comparisons.

The results of all statistical tests were considered significant if *P* values were $<.05$.

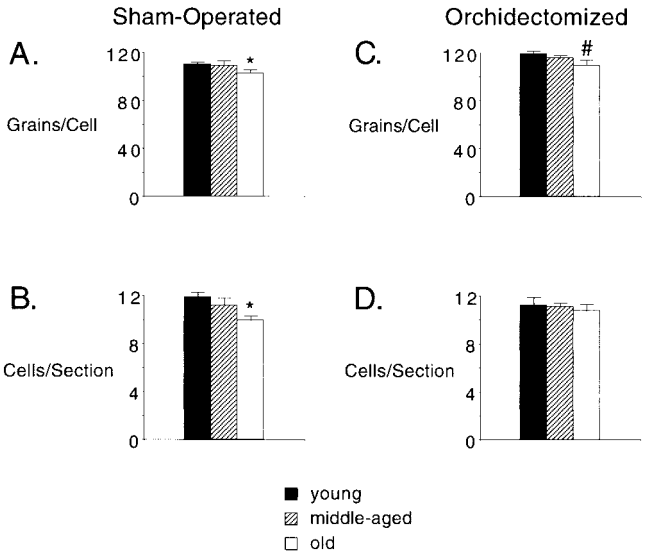

Figure 1. Prepro–gonadotropin-releasing hormone (ppGnRH) messenger RNA (mRNA) content measured by in situ hybridization histochemistry in the medial preoptic area (MPOA) and diagonal band of Broca (DBB) of sham-operated **(A, B)** and orchidectomized **(C, D)** young (3-month-old, solid bars; n = 8 and 8, respectively), middle-aged (13-month-old, hatched bars; n = 8 and 7, respectively), and old (23-month-old, open bars; n = 7 and 7, respectively) male Brown Norway rats. ppGnRH mRNA content was quantitated by measurement of the number of silver grains per cell **(A, C)** and the number of labeled cells per section **(B, D)**. Values shown are means ± SEM. The number of grains per cell was significantly reduced in old compared with young and middle-aged rats in both the sham-operated and orchidectomized groups. The number of labeled cells per section was significantly decreased in old compared with young and middle-aged sham-operated, but not orchidectomized, animals (* denotes $P < .05$ vs young and middle-aged rats; # denotes $P < .05$ vs young rats).

Results

ppGnRH mRNA Content (Study 1A)

In sham-operated BN rats, the cellular ppGnRH mRNA content in MPOA and DBB neurons (Figures 1A and 2) was significantly lower in old (24-month-old; 102.6 ± 2.8 grains/cell [mean ± SEM]) compared with young (3-month-old; 110.3 ± 1.9 grains/cell) and middle-aged (13-month-old; 109.1 ± 4.0 grains/cell) animals ($P < .05$). The number of MPOA and DBB neurons expressing the GnRH gene (Figures 1B and 2) decreased progressively with aging in sham-operated young (11.8 ± 0.4 cells/section), middle-aged (11.1 ± 0.6 cells/section), and old rats (9.9 ± 0.4 cells/section) ($P < .05$).

Similar results were obtained in orchidectomized rats for the cellular ppGnRH mRNA content (Figure 1C), which was decreased with aging in young (118.8 ± 2.8 grains/cell), middle-aged (115.9 ± 1.8 grains/cell), and old (109.6 ± 4.4 grains/cell; $P < .05$) rats. However, the number of MPOA and DBB neurons expressing the GnRH gene (Figure 1D) was unchanged with aging in orchidectomized young (11.2 ± 0.7 cells/section), middle-aged (11.1 ± 0.3 cells/section), and old rats (10.8 ± 0.5 cells/section).

The cellular ppGnRH mRNA content in MPOA and DBB neurons was significantly increased with orchidectomy in young (sham-operated: 110.3 ± 1.9 grains/cell; orchidectomized: 118.8 ± 2.8 grains/cell), middle-aged (sham-operated: 109.1 ± 4.0 grains/cell; orchidectomized: 115.9 ± 1.8 grains/cell), and old (sham-operated: 102.6 ± 2.8 grains/cell; orchidectomized: 109.6 ± 4.4 grains/cell) rats ($P < .05$). However, the number of MPOA and DBB neurons expressing the GnRH gene was unchanged with orchidectomy in young (sham-operated: 11.8 ± 0.4 grains/cell; orchidectomized: 11.2 ± 0.7 grains/cell), middle-aged (sham-operated: 11.1 ± 0.6 grains/cell; orchidectomized: 11.1 ± 0.3 grains/cell), and old (sham-operated: 9.9 ± 0.4 grains/cell; orchidectomized: 10.8 ± 0.5 grains/cell) rats.

In all age groups, the highest concentration of neurons expressing GnRH was found at the level of the organum vasculosum of the lamina terminalis and the rostral MPOA, with a marked decrease in the density of GnRH neurons rostral and caudal to this level. The effects of aging and orchidectomy on ppGnRH mRNA levels were

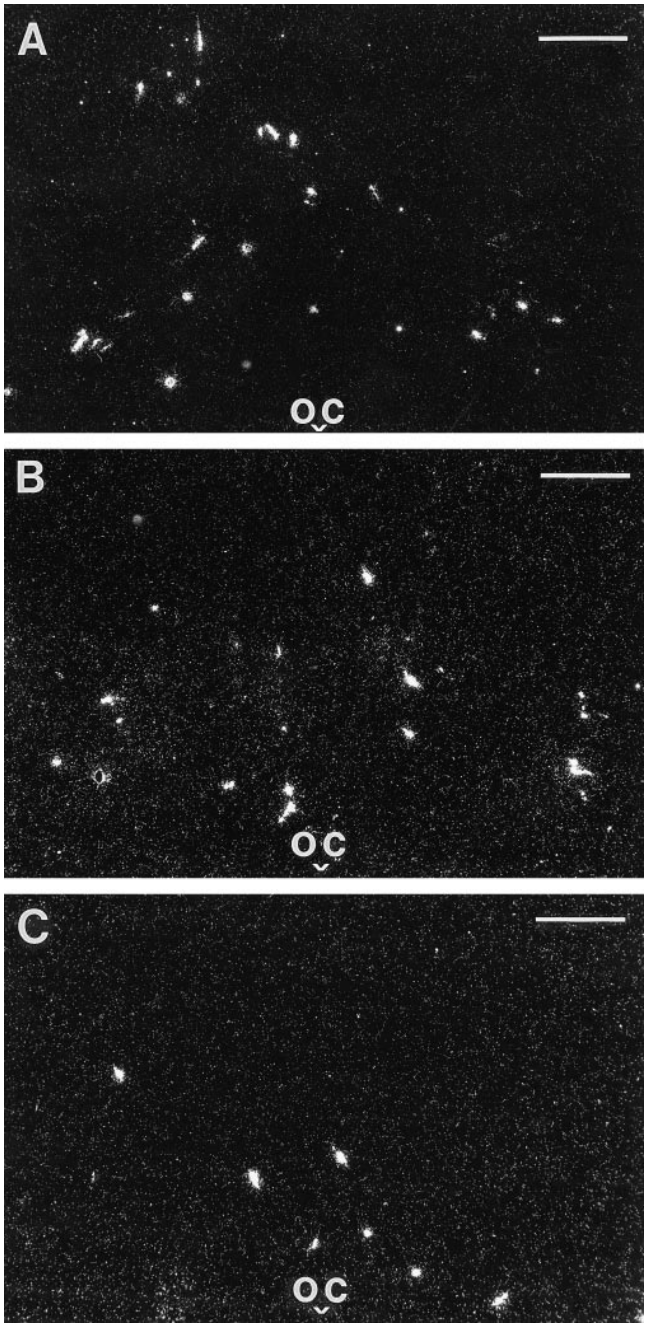

Figure 2. **(A)** Dark-field photomicrograph of a coronal brain section showing neurons containing prepro–gonadotropin releasing hormone (GnRH) messenger RNA labeled by in situ hybridization histochemistry from the rostral medial preoptic area (MPOA) of a sham-operated (intact) 3-month-old male Brown Norway rat. **(B, C)** Dark-field photomicrographs of anatomically matched sections from the rostral MPOA of sham-operated 13- and 23-month-old male Brown Norway rats, respectively. Note that the number of labeled cells is decreased in the old compared with the younger rats. Assessment of the number of silver grains per labeled cell is not possible at this magnification, however. ''oc'' denotes optic chiasm (below the field of view). Bar = 200 μm.

Serum testosterone and gonadotropin levels in intact and sham-operated male Brown Norway rats (study 1)

		Hormone* (ng/mL)		
Age of rats	n	T	LH	FSH
3 months	17–20	1.05 ± 0.18	0.39 ± 0.04	5.5 ± 0.5
13 months	19–20	0.85 ± 0.10	0.36 ± 0.03	6.2 ± 0.5
22–23 months	18–19	0.47 ± 0.06†	0.38 ± 0.03	7.6 ± 0.5‡

* T indicates testosterone; LH, luteinizing hormone; and FSH, follicle-stimulating hormone. Values are mean ± SEM.
† $P < 0.01$ compared with the 3-month-old group.
‡ $P < 0.05$ compared with the 3-month-old group.

not localized to any particular region of the MPOA or DBB (data on cellular ppGnRH mRNA content and the number of neurons expressing the GnRH gene within regions of the MPOA and DBB not shown).

Testosterone and Gonadotropin Levels in Intact and Sham-Operated Rats (Studies 1A and 1B)

In intact and sham-operated rats, serum T levels were decreased in old compared with young rats (Table). Serum LH levels were unchanged with aging; however, levels of FSH were significantly increased in old compared with young animals.

GnRH Peptide Content in Intact Rats (Study 1B)

GnRH peptide content was significantly decreased in the MPOA (Figure 3A) of intact middle-aged (50 ± 3 pg/mg protein) and old (45 ± 4 pg/mg protein) compared with young animals (73 ± 5 pg/mg protein; $P < .01$ vs middle-aged and old). In the ME, GnRH content decreased progressively from young (23.2 ± 1.6 ng/mg protein) to middle-aged (20.1 ± 1.9 ng/mg protein) to old (15.8 ± 2.3 ng/mg protein; $P < .05$ vs young) rats (Figure 3B). A trend toward decreased GnRH content was observed in the ARC from young (1.4 ± 0.2 ng/mg protein) to middle-aged (1.3 ± 0.3 ng/mg protein) to old (1.1 ± 0.3 ng/mg protein) rats (Figure 3C), but this change was not significant.

Gonadotropin and T Responses to GnRH Stimulation in Intact BN Rats (Study 2)

The LH response to GnRH stimulation was not significantly affected by aging (Figure 4A). The AUC was not significantly changed with aging (young: 81.7 ± 9.6 ng/mL/min; middle-aged: 75.5 ± 9.6 ng/mL/min; old: 84.4 ± 11.6 ng/mL/min), nor were there any differences between age groups in the increment in LH levels from baseline (time 0) to peak response after GnRH administration (young: 1.6 ± 0.2 ng/mL; middle-aged: 1.5 ± 0.2 ng/mL; old: 1.7 ± 0.2 ng/mL).

In contrast to LH, FSH responsiveness to GnRH tended to increase with aging (Figure 4B). The AUC for FSH tended to be higher in old than in younger rats (young:

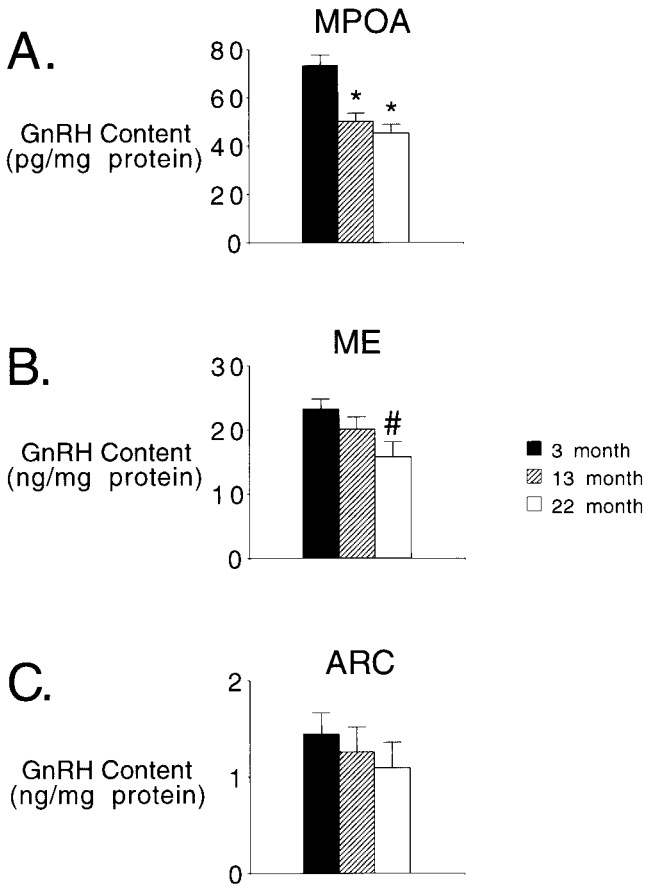

Figure 3. Gonadotropin releasing hormone (GnRH) peptide content measured by radioimmunoassay in the medial preoptic area (MPOA) **(A)**, median eminence (ME) **(B)**, and arcuate nucleus (ARC) **(C)** of intact young (3-month-old, solid bars; n = 12), middle-aged (13-month-old, hatched bars; n = 12), and old (22-month-old, open bars; n = 11) male Brown Norway rats. Values shown are means ± SEM. GnRH content was significantly reduced in the MPOA of old and middle-aged compared with young animals (* denotes $P < .01$ vs young). In addition, GnRH content was lower in the ME of old compared with young rats (# denotes $P < .05$ vs young). In the ARC, GnRH content tended to decrease with aging, but this change was not significant.

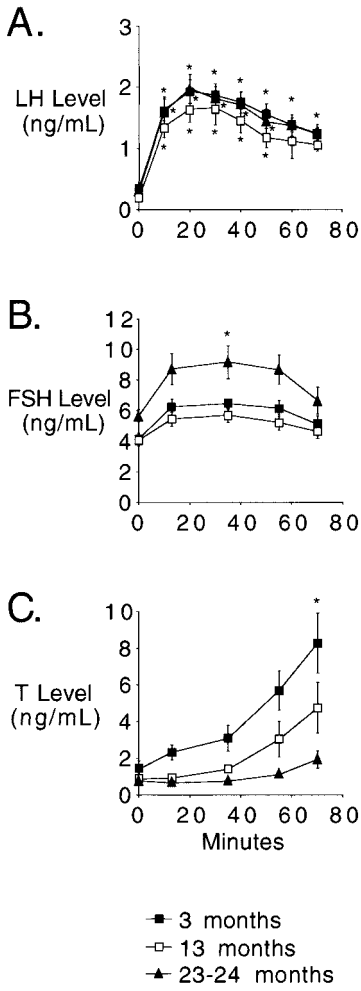

Figure 4. Serum luteinizing hormone (LH) **(A)**, follicle-stimulating hormone (FSH) **(B)**, and testosterone (T) **(C)** levels measured by radioimmunoassay at baseline and at 10-minute intervals after intravenous injection of 100 ng/100 g body weight of gonadotropin-releasing hormone (GnRH) in intact young (3-month-old, filled squares; n = 9), middle-aged (13-month-old, open squares; n = 9), and old (23- to 24-month-old, filled triangles; n = 10) male Brown Norway rats. Values shown are means ± SEM (* denotes $P < .05$ vs 0 minutes). Because of insufficient sample volumes, FSH and T samples were pooled into groups around the median time points of 13, 35, and 55 minutes after GnRH. LH responses (area under the curve [AUC] or increment compared to time 0) to GnRH were not significantly different between young, middle-aged, or old rats, whereas FSH responsiveness to GnRH tended to increase with aging (AUC and increment compared to time 0: $P < .05$ for old compared with middle-aged rats). Finally, with aging, the T response to GnRH was decreased (AUC: $P < .01$ for old vs young; increment compared to time 0: $P < .05$ for old vs young) and progressively delayed.

127.3 ± 16.0 ng/mL/min; middle-aged: 82.8 ± 12.3 ng/mL/min; old: 187.8 ± 41.1 ng/mL/min; $P < .05$ for old vs middle-aged), and the increment in FSH levels from baseline to peak response also tended to increase with aging (young: 2.6 ± 0.3 ng/mL; middle-aged: 1.6 ± 0.2 ng/mL; old: 3.5 ± 0.7 ng/mL; $P < .05$ for old vs middle-aged), although for both of these methods of determining the FSH response, the effect of aging was significant only for the 13-month vs the 23–24-month comparison.

The T response to GnRH stimulation (Figure 4C) was diminished with aging. The AUC decreased with aging, from 167 ± 47 ng/mL/min in young rats to 79 ± 32 ng/mL/min in middle-aged rats to 11 ± 8 ng/mL/min in old rats ($P < .01$ for young vs old). Furthermore, the increment in T levels from time 0 to peak response after GnRH administration was diminished with aging, reaching 6.3 ± 1.6 ng/mL in young rats at 70 minutes after GnRH, compared with 3.8 ± 1.4 ng/mL in middle-aged and 1.3 ± 0.5 ng/mL in old rats ($P < .05$ for young vs old).

Circadian Variation in Serum LH, T, and B Levels (Study 3)

Serum LH levels over a 24-hour period were significantly decreased with aging (Figure 5A), as was evidenced by

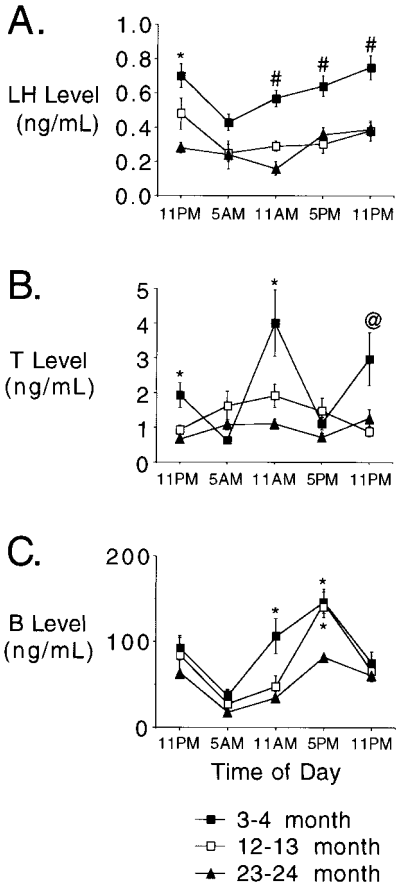

Figure 5. Circadian variation in serum luteinizing hormone (LH) **(A)**, testosterone (T) **(B)**, and corticosterone (B) **(C)** levels measured by radioimmunoassay in intact young (3- to 4-month-old, filled squares), middle-aged (12- to 13-month-old, open squares), and old (23- to 24-month-old, filled triangles) male Brown Norway rats sacrificed at 6-hour intervals over a 24-hour period (n = 8 to 9/time point for each age group). Values shown are means ± SEM (* denotes $P < .05$ vs old; # denotes $P < .01$ vs middle-aged and old; @ denotes $P = .05$ vs old). Note that the levels of all 3 hormones measured over a 24-hour period showed a significant and progressive reduction with aging. In addition, T levels exhibited a bimodal diurnal variation in young but not in older rats, and B levels exhibited a circadian rhythm with a decreasing amplitude in old compared with younger rats.

a significant reduction in the AUC for LH in older animals (young: 14.2 ± 0.7 ng/mL/h; middle-aged: 7.5 ± 0.5 ng/mL/h; old: 6.6 ± 0.6 ng/mL/h; $P < .01$ for young vs middle-aged and old rats). There appeared to be a circadian variation in LH levels, with the lowest LH levels in the morning hours and the highest levels late in the evening. The amplitude of this apparent circadian rhythm, as determined by the differences between peak and baseline LH levels over a 24-hour period, tended to decrease with aging (young: 0.49 ± 0.5 ng/mL; middle-aged: 0.43 ± 0.7 ng/mL; old: 0.38 ± 0.5 ng/mL), but this change was not significant.

T levels over a 24-hour period were significantly decreased with aging (Figure 5B), with a progressive reduction in the AUC from young (45.4 ± 7.6 ng/mL/h) to middle-aged (31.4 ± 4.3 ng/mL/h) to old rats (19.5 ± 2.1 ng/mL/h; $P < .01$ for young vs old animals). T levels showed an apparent circadian variation in young rats, although this variation reflected a bimodal pattern with a major peak in the late morning ($P < .05$ for 1100-hour vs 0500-hour T level) and a smaller peak in the late evening, rather than a unimodal pattern, as for B and possibly LH. This bimodal diurnal variation was not evident in middle-aged or old rats, and the degree of this variation, as determined by the differences between peak and baseline T levels over a 24-hour period, was progressively blunted with aging (young: 3.9 ± 0.7 ng/mL; middle-aged: 1.7 ± 0.4 ng/mL; old: 0.8 ± 0.2 ng/mL; $P < .01$ for young vs middle-aged and old rats). For confirmation, serum samples were retested in a separate T assay, and a similar pattern of circadian variation was observed (data not shown).

B levels over a 24-hour period were significantly and progressively decreased with aging (Figure 5C), as was evidenced by a decrease in the AUC from 2236 ± 199 ng/mL/h in young rats to 1773 ± 115 ng/mL/h in middle-aged rats to 1181 ± 68 ng/mL/h in old animals ($P < .01$ for young vs old; $P < .05$ for middle-aged vs old). B showed a definite circadian rhythm with a late afternoon peak in all 3 age groups, but the amplitude of the rhythm, as determined by the differences between peak and baseline B levels over a 24-hour period, was decreased in old compared with younger rats (young: 124 ± 15 ng/mL; middle-aged: 139 ± 15 ng/mL; old: 76 ± 4 ng/mL; $P < .05$ for young vs old; $P < .01$ for middle-aged vs old rats).

Discussion

We previously reported evidence that male BN rats develop both primary and secondary (hypothalamic/pituitary) testicular failure with aging, similar to that seen in human males (Gruenewald et al, 1994a). In this report, we have further characterized age-related alterations in hypothalamic/pituitary function in these animals, complementing and adding to a body of earlier work on aging male BN rats (Wang et al, 1993; Zirkin et al, 1993; Chen et al, 1994; Bonavera et al, 1997, 1998). Using ISHH, we found a decrease in cellular ppGnRH mRNA levels and in the number of cells expressing the GnRH gene with aging in intact (sham-operated) rats. Furthermore, GnRH content was decreased with aging in the MPOA and the ME of intact male BN rats, which contain the perikarya and the terminal neurosecretory boutons of GnRH neurons, respectively. Taken together, the age-related reductions in ppGnRH mRNA levels and GnRH peptide content indicate a reduction in GnRH synthetic capacity in aging male BN rats. We also found that cellular ppGnRH mRNA levels were decreased with aging in orchidecto-

mized rats, which suggests that the age-related decrease in GnRH gene expression is not dependent upon testicular feedback factors. Finally, the LH response to GnRH stimulation was unchanged, and the FSH response tended to increase with aging, thus indicating intact pituitary responsiveness to GnRH with aging. Collectively, these observations suggest that secondary testicular failure in the aging male BN rat is due to altered hypothalamic rather than pituitary function.

We found that in male Fischer 344 (F344) rats, pp-GnRH mRNA and gonadotropin levels are decreased with aging in intact but not in orchidectomized animals (Gruenewald and Matsumoto, 1991a). However, these findings were confounded by excessive circulating progesterone concentrations from testicular Leydig cell tumors, which we subsequently demonstrated in old F344 rats (Gruenewald et al, 1992). Specifically, elevated progesterone and other sex steroids in these old F344 rats may have suppressed GnRH and gonadotropin secretion and contributed to the age-related secondary hypogonadism in those rats. In contrast to the F344 rat, male BN rats do not develop testicular Leydig cell tumors or excessive sex steroid secretion with aging (Gruenewald et al, 1994a), and we found an age-related decrease in ppGnRH mRNA in both intact (sham-operated) and orchidectomized BN rats. Therefore, unlike the F344 rat, the age-related decrease in GnRH gene expression in the BN rat is independent of testicular feedback factors. Based on the foregoing discussion and on previous work by our group and others (Wang et al, 1993; Zirkin et al, 1993; Gruenewald et al, 1994a), we believe that alterations in hypothalamic regulation of reproductive axis function in aging male BN rats are more relevant to the situation in aging men than are our earlier findings in the F344 rat.

A number of previous studies have evaluated the effect of orchidectomy on ppGnRH mRNA levels in young rodents, and they have shown conflicting results (Rothfeld et al, 1987; Park et al, 1988; Toranzo et al, 1989; Wiemann et al, 1990; Selmanoff et al, 1991), which suggests that the effect of testicular feedback factors, such as T, on GnRH gene expression is not robust in young animals. In our study, cellular ppGnRH mRNA content was significantly increased with orchidectomy in young BN rats, but the number of cells expressing the GnRH gene actually tended to be slightly lower in orchidectomized than in sham-operated rats, which suggests that there is little net overall effect of orchidectomy on GnRH gene expression in young rats. However, with aging, both indices of GnRH gene expression increased with orchidectomy, which suggests that testicular factors may play a greater role in regulating GnRH gene expression in old than in young animals. This increased effect of orchidectomy on GnRH gene expression with aging may be due in part to an increased sensitivity to T negative feedback with aging. A number of studies in male rats (Shaar et al, 1975; Pirke et al, 1978; Gray et al, 1980) and in human males (Winters et al, 1984; Deslypere et al, 1987) have demonstrated that the suppressive effect of androgenic feedback on serum gonadotropin levels increases with aging. Furthermore, androgens exert a greater inhibitory effect on gonadotropin pulse frequency in older than in younger men (Winters et al, 1984), which suggests increased negative feedback suppression on the hypothalamic GnRH pulse generator. Thus, increased sensitivity to T feedback may contribute to greater suppression of GnRH gene expression in intact old BN rats compared with younger BN rats, even though absolute T levels are decreased with aging.

The effects of aging upon ppGnRH mRNA levels that we observed in sham-operated male BN rats (a 20 to 25% decrease with aging, if changes in both the number of GnRH-expressing neurons and the cellular ppGnRH mRNA content are considered together) are relatively modest in comparison to the effects of aging on GnRH peptide content in the MPOA and ME (35 to 40% lower GnRH content in old rats). This suggests that factors other than altered GnRH gene expression may contribute to age-related impairment in hypothalamic/pituitary regulation of reproductive axis function. Other potential influences on hypothalamic/pituitary regulation that may be affected by aging include posttranslational processing of GnRH and changes in other neurotransmitters and neuropeptides that affect GnRH and gonadotropin secretion, such as dopamine, neuropeptide Y, and the endogenous opioid peptides. Alternatively, it is possible that a small effect of aging on GnRH gene expression may exert a disproportionate effect on GnRH release into the hypothalamic-pituitary portal circulation and, in turn, on gonadotropin secretion. Finally, decreases in GnRH gene expression within the smaller subset of GnRH neurons that project to the ME and that directly stimulate gonadotropin secretion could be greater than that observed in the entire population of GnRH neurons.

A number of investigators have measured gonadotropin levels after GnRH infusion to determine whether impaired pituitary gonadotropin secretion contributes to age-related reproductive insufficiency. Some of these studies in male rat models (such as Sprague-Dawley or Long-Evans rats) suggested decreased pituitary gonadotropin responsiveness to GnRH with aging (Riegle and Meites, 1976; Bedrak et al, 1983). However, even in these rats, the defect appeared to be remediable, as evidenced by the restoration of gonadotropin levels to young adult levels after priming of the pituitary by multiple GnRH injections, which suggests chronic hypothalamic GnRH deficiency or understimulation (Miller and Riegle, 1978). In BN rats, we found that gonadotropin responses to GnRH were unimpaired in old animals even without priming, suggesting

intact pituitary function with aging. Our results are similar to the findings of Bonavera et al, who also reported no change in the gonadotropin response to GnRH administration with aging, although in their study, the T response to GnRH was not reported (Bonavera et al, 1998). These findings in aging male BN rats are analogous to those in aging adult males, who exhibit unimpaired LH responses to intravenous GnRH administration (Kaufman et al, 1991). However, we did not perform a dose-response study to evaluate gonadotropin responsiveness to a range of GnRH doses. Therefore, we were unable to exclude the possibility that older BN rats have impaired pituitary responsiveness to doses of GnRH that are smaller than those employed in our study.

It is noteworthy that although LH responsiveness to GnRH was similar in rats of different ages, FSH responsiveness to GnRH tended to increase with aging. Furthermore, baseline LH levels were unchanged with aging (or were decreased when measured over 24 hours), in contrast to the increase in basal FSH levels with advancing age. The reasons for the dissociation of aging effects on LH and FSH in BN rats are unknown. It is intriguing to speculate that this dissociation may involve differential testicular feedback effects on LH and FSH with aging. In any event, in light of our findings that suggest reduced GnRH synthetic capacity with aging, the apparent increase in FSH responsiveness to GnRH in old rats, perhaps due both to reduced T and inhibin B concentrations (Wang et al, 1993), seems to be sufficient to result in increased baseline FSH levels in the face of decreased GnRH stimulation.

Despite similar LH responses to GnRH, the testicular response to LH decreased progressively with aging after GnRH stimulation. In the presence of similar LH response kinetics and LH levels induced by GnRH, young rats exhibited increased T levels by 10 minutes after GnRH administration, whereas with advancing age, the increment in T levels was increasingly delayed. Because blood sampling ended at 70 minutes after intravenous GnRH administration and because serum T levels seemed to be continuing to increase in all age groups at that time point, it is unknown whether T levels would ultimately have reached the same level in older as in younger rats. However, the slope of the increase in T levels after GnRH administration became progressively flattened with aging, which suggests that the eventual maximum T level would also have been lower in older rats. It is noteworthy that in middle-aged rats, T levels tended to be lower than those seen in young rats—both at baseline and after GnRH stimulation. This suggests the existence of a subtle defect in testicular function, one that occurs by middle age. In combination with earlier findings of decreased spermatogenesis and decreased T levels in old rats despite stable to increased gonadotropin levels (Wang et al, 1993; Gruenewald et al, 1994a) and other work indicating reduced Leydig cell T production in aging BN rats (Chen et al, 1994), these data provide further evidence of progressive primary testicular failure in old male BN rats.

We observed a bimodal diurnal variation in T levels in young but not in older rats, with a major peak in T levels in the late morning and a smaller peak in the late evening. This diurnal rhythmicity is similar to a trimodal variation in T levels, which has been reported in young adult Sprague-Dawley rats (Mock et al, 1978), although another group reported a unimodal diurnal variation in young adult but not in old rats of this strain (Simpkins et al, 1981). Like Bonavera et al, we found that T levels were higher in the late morning than in the late afternoon in young but not in older male BN rats (Bonavera et al, 1997). However, this group did not sample blood between 1800 and 1000 hours; therefore, corroboration of the late-evening surge in T levels that we found in young BN rats is currently unavailable. As reported in other rat strains (Wong et al, 1983), we found peak plasma levels of B (the major circulating adrenal corticosteroid hormone in the rat) in the early evening (in contrast to humans, in whom peak cortisol levels occur around 0800 hours). We observed a significant blunting of the amplitude of the B circadian rhythm with aging. Our observations of apparent dampening of T and B circadian rhythms with aging in the BN rat are analogous to the changes in rhythms of these hormones in aging humans (Tenover et al, 1988; van Coevorden et al, 1991), and they probably reflect age-related alterations in hypothalamic regulation of T and B secretion.

An alternative explanation for the apparent bimodal diurnal variation in T levels that we observed in young rats is pulsatile T secretion, which is well described in young adult male rats (Heywood, 1980; Ellis and Desjardins, 1982; Veldhuis et al, 1987). We found that the T levels within groups of animals at each time point over a 24-hour period were quite variable, especially in young rats, which suggests that T pulses in individual rats may have contributed to variations in T levels over a 24-hour period in young compared with older rats. Furthermore, the attenuation in the bimodal diurnal variation that we found in older rats may reflect a decrease in the amplitude of pulsatile T secretion with aging. Although they did not subject their findings to formal analysis, Bonavera et al found evidence of pulsatile T secretion in individual young rats, and the pulse amplitude was decreased in the representative old rats used for comparison (1997). However, our findings of a major peak in T levels at 1100 hours and a trough at 1700 hours in young rats are similar to the results reported by Bonavera et al (1997), which suggests that we were observing a true circadian rhythm. Demonstration of the expected circadian variation in serum B levels in young animals suggests that stress did

not contribute to the variations in serum T levels in these animals.

Although serum LH levels at a single time point were unchanged in study 1, when assessed over a 24-hour period in study 3, a significant age-related decline was detected. These findings over a 24-hour period could be explained by LH circadian rhythmicity that diminishes with aging, and they suggest that a difference in LH levels between age groups might be detected at one time of day but not at another. This interpretation is supported by our finding of an apparent LH circadian rhythm in young rats that tended to decline with aging (Figure 5A), although we were unable to show a statistically significant difference across ages in the amplitude of LH circadian rhythmicity over a 24-hour period. Based on these observations, the time of day may be an important factor in the measurement of LH levels in these rats, and it may explain our failure to detect a decrease in LH levels with aging using measurements performed at a single time point in study 1.

We observed an age-related decline in serum B levels and a blunting of the circadian variation of serum B. Numerous studies have reported age-related changes in basal glucocorticoid levels in rodents. Some investigators find that basal B levels increase with aging, whereas others do not. In a review of over 20 studies, Sapolsky was unable to attribute this inconsistency between studies to strain differences or the point in the circadian cycle at which samples were obtained (Sapolsky, 1992). Instead, the most important variable seemed to be the degree of stress experienced by study animals prior to obtaining the samples. Studies reporting no age-related increase in B tended to find elevated basal B levels in young rats. Average B levels in our young animals ranged between 50 and 150 ng/mL, values comparable to those obtained by other investigators in unstressed rats, which indicates that our animals did not experience significant stress prior to sample collection. Furthermore, our findings are consistent with other studies in male BN rats, in which basal B levels were either unchanged or tended to decrease with aging (van den Berg et al, 1991; van Eekelen et al, 1991), which suggests that basal B levels may not increase with aging in male BN rats. Plasma B levels also decline with aging in F344/BN F1 hybrid rats (Cizza et al, 1994; Hauger et al, 1994). Finally, we did not measure plasma adrenocorticotropin hormone (ACTH) levels; therefore, it is unknown whether the low basal B levels we observed in older rats were appropriate for the level of ACTH stimulation compared with young rats. Studies involving concomitant ACTH measurements and measurements under both baseline and stressful conditions have been performed by others (van den Berg et al, 1991; van Eekelen et al, 1995), and these studies are important in assessing the suitability of the aging male BN model for the study of hypothalamic/pituitary/adrenal axis aging.

Although we have demonstrated a decrease in GnRH gene expression with aging in male BN rats, it is unclear whether these changes are primarily due to intrinsic alterations in GnRH neurons with reduced GnRH synthetic capacity or to changes in other neurotransmitters, neuropeptides, or neuromodulators affecting reproductive axis function (eg, neuropeptide Y [NPY], catecholamines, excitatory or inhibitory amino acids, and endogenous opioid peptides). We previously demonstrated a decrease in prepro-NPY mRNA levels with aging in these rats, suggesting that decreased stimulatory NPY activity may contribute to altered reproductive function in older BN rats (Gruenewald et al, 1994b). However, neurons producing a number of other neuromodulatory substances also synapse onto GnRH neurons that project to the median eminence. For example, Vernet et al recently demonstrated an age-related increase in inducible nitric oxide synthase (iNOS) in the hypothalamus of BN rats, and nitric oxide is thought to play an important role in mediating the stimulatory effects of glutamatergic neurons on GnRH release (Vernet et al, 1998). It is possible that increased brain levels of iNOS may lead to neurotoxicity, which, in turn, may contribute to the age-related impairment in GnRH secretion. The relative importance of changes in these and other neurotransmitters and neuromodulators in mediating age-related impairment of reproductive axis regulation has not yet been determined.

In summary, our studies in aging male BN rats show that both GnRH gene expression and GnRH content in the hypothalamus are decreased with aging but that pituitary responsiveness to GnRH administration is unimpaired with aging. These findings imply that the secondary testicular failure in these rats is due to a reduction in hypothalamic GnRH synthetic capacity rather than to a pituitary problem. In addition, we found evidence of altered circadian rhythmicity for both T and B, suggesting altered hypothalamic regulation of both gonadal and adrenal axis function with aging in these animals. Finally, we found that the age-related decrease in GnRH gene expression in male BN rats occurred independently of testicular feedback factors. Further work is needed to clarify whether age-related alterations in GnRH synthetic capacity are due to an intrinsic impairment in GnRH neuronal function or to alterations in other neurotransmitters, neuropeptides, and hormones that affect GnRH function. The male BN rat appears to be an ideal model for pursuing these and other questions regarding the nature of male reproductive aging.

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