Aging attenuates both the regularity and joint synchrony of LH and testosterone secretion in normal men: analyses via a model of graded GnRH receptor blockade

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Liu, Peter Y., Steven M. Pincus, Paul Y. Takahashi, Pamela D. Roebuck, Ali Iramnaneh, Daniel M. Keenan, and Johannes D. Veldhuis. Aging attenuates both the regularity and joint synchrony of LH and testosterone secretion in normal men: analyses via a model of graded GnRH receptor blockade. Am J Physiol Endocrinol Metab 290: E34–E41, 2006; doi:10.1152/ajpendo.00227.2005.—Testosterone (T) secretion declines in the aging male, albeit for unknown reasons. From an ensemble perspective, repeated incremental signaling among gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and T is required to maintain physiological androgen availability. Pattern-regularity statistics, such as univariate approximate entropy (ApEn) and bivariate cross-ApEn, provide specific and sensitive model-free measurement of altered multipathway control. The present study exploits partial muting of one pathway (GnRH drive) to appraise adaptive regulation of LH and T secretion in young and aging individuals. Analyses comprised 100 paired 18-h LH and T concentration time series obtained in 25 healthy men ages 20–72 yr each administered placebo and three graded doses of a specific GnRH-receptor antagonist. Graded blockade of GnRH drive increased the individual regularity of LH and T secretion and the synchrony of LH-T feedforward and T-LH feedback in the cohort as a whole (P < 0.001 for each). However, age markedly attenuated ganirelix-induced enhancement of univariate T orderliness and bivariate LH-T feedback and T-LH feedback synchrony (P ≤ 0.0025). In summary, the present analyses support the thesis that aging disrupts coordinate control of T secretion, LH-T feedforward, and T-LH feedback in healthy men. Thus the experimental strategy of stepwise silencing of an agonistic pathway may have utility in dissecting the bases of altered neurohormonal linkages in other systems.

gonadotropin-releasing hormone; gonadotropin; aging; male; androgen; secretion; luteinizing hormone

AGING IS MARKED by a 30–50% decline in systemic testosterone (T) concentrations in healthy community-dwelling men, as recognized nearly 50 yr ago (17, 69). Subsequent population-based cross-sectional and longitudinal studies have corroborated relative androgen depletion in the aging male (1, 3, 7, 10, 15, 29, 33, 58, 60). Epidemiological analyses have correlated hypoandrogenemia with insulin resistance, dyslipidemia, sarcopenia, osteopenia, reduced physical stamina, sexual dysfunction, impaired quality of life, and (possibly) depressive mood and cognitive impairment (14, 32, 54, 61, 64, 81). Recent androgen-supplementation trials suggest that reduced T availability may contribute to frailty in older men (18, 26, 30, 41, 52, 57).

In complementation of therapeutic studies, mechanistic investigations are needed to determine the primary basis of aging-associated hypoandrogenemia. An evolving regulatory concept is that physiological T concentrations are determined by repeated incremental interactions among hypothalamic gonadotropin-releasing hormone (GnRH), pituitary luteinizing hormone (LH), and gonadal T, as mediated via reciprocal feedforward [GnRH → LH and LH → T] and feedback [T → GnRH and T → LH] signaling (21–25, 63, 69). Ensemble control of the GnRH-LH-T axis definitionally limits facile interpretation of experiments based exclusively upon isolating any one component of the axis (5, 11, 12, 19, 20, 31, 35–37, 56, 59, 63, 69, 79, 80).

Few validated noninvasive methodologies are available to quantify integrative regulation of complex, time-delayed, nonlinearly coupled biologically adaptive systems. Direct investigation of the network properties of an interlinked axis would in principle require simultaneous measurements of all input and output signals over time without disturbing their ongoing interactions. An alternative experimental approach comprises simultaneous monitoring of two or more key input and output signal trains followed by application of suitable ensemble statistics, such as approximate entropy (ApEn; a regularity measure) and cross-ApEn (a joint synchronicity metric; see Refs. 42 and 49). Both statistics have high sensitivity and specificity (>90%) in reductionistic mathematical models and multipathway neuroendocrine systems (16, 43, 45, 46, 48, 74). Univariate ApEn discriminates subtle degrees of underlying process randomness, varying from maximally irregular to highly ordered in a scale- and model-independent manner (42, 47). Scale invariance ensures reliable comparisons in the face of potentially disparate hormone concentrations, and model independence allows valid contrasts between processes driven by variable (and unknown) admixtures of underlying deterministic and stochastic factors. Bivariate cross-ApEn quantifies the pairwise synchrony (relative regularity) of interlinked signals, such as coupled numerical and hormonal time series (48, 49).
Extension of this concept to include forward and reverse cross-ApEn permits synchrony discrimination for directionally defined forward and reverse two-signal linkages, e.g., feedforward (stimulatory) regulation of T secretion by LH and feedback (inhibitory) control of LH secretion by T, and analogously for reciprocal signaling between ACTH and cortisol (27, 28).

The present investigation tests the hypothesis that aging impairs one or more linkages among GnRH, LH, and T. To this end, the experimental paradigm comprised partial suppression of feedforward drive by GnRH, achieved by administration of graded doses of a competitive GnRH-receptor antagonist in 25 healthy men ages 20–72 yr. Each stratum of LH and T output thereby constituted a model of time-varying feedforward/feedback adaptations to controlled muting of a single agonistic pathway. We postulated that, under these experimental conditions, model- and scale-independent quantification of individual LH and T secretory regularity and joint LH → T and T → LH synchrony would unveil age-related attenuation of specific pathway control.

METHODS

Sampling Protocol

Twenty-five men aged 20–72 yr (mean 43 yr, 2–5 men/decade) participated in the study after providing written voluntary informed consent approved by the Mayo Clinic Institutional Review Board. Participants were healthy community-dwelling men within 20% of ideal body weight, who had not undertaken recent transmeridian travel (within 10 days) or consumed alcohol, caffeine, or systemic medications within five biological half-lives. Detailed medical inventory excluded a history of infertility, systemic disease, recent weight change (exceeding 2 kg in the preceding 6 wk), T therapy, or psychoactive drug use. Outpatient screening was unremarkable in relation to medical history (particularly libido and erectile function), physical examination (including testis size), and fasting morning (0800) biochemical tests of renal, hepatic, hematological, endocrine, and metabolic function (plasma glucose and serum thyroxine, prolactin, insulin-like growth factor I, LH, follicle-stimulating hormone, T, and estradiol).

Volunteers were admitted to the Mayo General Clinical Research Center for four separate overnight inpatient studies scheduled at least 7 days apart. In each study session, one dose of 0 (saline), 0.1, 0.3, or 1.0 mg/m² ganirelix was administered subcutaneously at 2000 in a prospectively randomized double-blind fashion. Blood samples (1.0 ml) were withdrawn every 10 min beginning at 1800 for a total of 18 h through a forearm intravenous catheter for later assay of serum LH and T concentrations. Blood was allowed to clot at room temperature, and sera were frozen at −20°C.

GnRH receptor antagonist. Ganirelix is a potent, specific, and competitive GnRH receptor antagonist used in ovulation induction protocols (39, 40, 60a). Time course studies in young men have indicated that single administration of ganirelix (2.0 mg) suppresses pulsatile LH and T secretion by >80% in 4–8 h and maintains comparable suppression for an additional 16–20 h (67). The duration of inhibition is consistent with the plasma half-life of ganirelix of 15 ± 2 h. Unlike agonists, selective antagonists of GnRH action do not directly inhibit gonadal function (60a, 67, 76).

Assays

LH and T concentrations were measured in duplicate by automated immunochemiluminometry (ACS Corning, Bayer, Tarrytown, NY; see Ref. 77). The LH standard was derived from the Second International Reference Preparation. Concentration-dependent intra-assay coefficients of variation (CVs) averaged 5.5, 4.7, 3.5, and 3.8% and interassay CVs 6.5, 5.2, 3.7, and 4.7% at LH concentrations of 1.3, 4.4, 18, and 38 IU/l, respectively. Procedural sensitivity was 0.05 IU/ml. T was quantitated in the same assay system, wherein median intra- and interassay CVs were 6.8 and 8.3%, respectively, and the sensitivity was 18 ng/dl. T measurements obtained by immunochemiluminometry correlate strongly (r² = 0.98, slope 1.07) with those determined by gas chromatography-mass spectrometry (8).

Blood ganirelix concentrations were measured in duplicate by in-house RIA using polyclonal rabbit antisera (Anaspec, San Jose, CA), as previously described (38). The antisera does not cross-react with native GnRH at concentrations ranging from 30 to 1,000 ng/ml. Ganirelix was labeled via the chloramine-T reaction. Incubations are conducted at an antibody dilution of 1:3,000 in RIA buffer (0.1 M phosphate buffer, pH 7.4, 0.8% NaCl, 0.5% BSA, 0.01% thimerosal, 0.01% Triton X-100, and 0.1 mM EDTA; see Ref. 38). Bound and free ligand are separated by precipitation with goat antirabbit antisera. Mean intra-assay CVs were 8.9, 5.7, and 18.7% and interassay CVs were 8.2, 4.0, and 9.2% at 0.5, 1.0, and 10 ng/ml, respectively. Assay sensitivity was 0.05 ng/ml.

ApEn and Cross-ApEn

The ApEn statistic quantifies the degree of irregularity, or disorderliness, of a single time series (42). Technically, ApEn is defined as the summed logarithmic likelihood that templates (of length m) of patterns in the data that are similar (within r) remain similar (within the same tolerance r) on next (m ± 1) incremental comparison, as validated elsewhere (46, 47). ApEn of any given time series is a single nonnegative number, which confers an ensemble estimate of relative process randomness wherein larger ApEn values denote greater irregularity.

Cross-ApEn. Cross-ApEn quantifies joint pattern synchrony between two separate but linked sequences (43, 48, 49, 51), as formally defined (49). For the present 18-h hormonal time series, ApEn and cross-ApEn calculations assumed r = 20% of the SD of each data set and m = 1, and for 6-h subsets r = 35% also with m = 1. These parameters afford sensitive, specific, valid, and statistically well-replicated measures of ApEn (16, 44, 45, 65, 73) and cross-ApEn (48, 51, 72).


Statistics

Repeated-measures one-way ANOVA was used to test the postulate that antagonism of GnRH action by increasing doses of ganirelix (4 factors) enhances LH or T regularity and joint LH-T and T-LH synchrony. The primary hypothesis that age attenuates physiological adjustments to graded inhibition of GnRH feedforward on LH and T regularity and their joint synchrony was assessed in three ways. First, linear regression analysis was applied to evaluate the relationship between ApEn or cross-ApEn and age at any given ganirelix dose. Significance was assessed by Pearson’s correlation coefficient. Second, the logarithm of ApEn or cross-ApEn was regressed linearly on ganirelix dose in each volunteer to estimate a slope (ganirelix sensitivity). The resultant 25 slopes were regressed linearly on age to examine the influence of age on ganirelix sensitivity. Third, two-way ANCOVA was employed to assess the individual and combined impact of ganirelix dose and age on ApEn and cross-ApEn, wherein the response to saline (0 dose ganirelix) served as the within-subject covariate. To explore the time course of feedback/feedforward changes, the same analyses were applied arbitrarily to the first, middle, and last 6-h blocks of the 18-h time period.

Analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). Repeated-measures ANOVA and ANCOVA were mod-
eled by Proc Mixed using a compound symmetrical correlation structure. Data are presented as means ± SE. *P* < 0.05 was construed as significant.

**RESULTS**

Figure 1 shows that escalating doses of ganirelix decrease all four individual LH ApEn, T ApEn, feedforward LH-T cross-ApEn, and feedback T-LH cross-ApEn assessed over the 18-h sampling interval (*P* < 0.0001, for each comparison). Decreased ApEn and cross-ApEn denote enhanced secretory regularity and joint synchrony, respectively. In the cohort as a whole, differential cross-ApEn was significantly negative (−0.17 ± 0.02, *P* < 10⁻⁵, signed ranks test), signifying less coordination of T-LH feedback than LH-T feedforward linkages. Differential cross-ApEn did not change with ganirelix dose (*P* = 0.38, not shown), indicating that blockade of GnRH drive augments feedforward and feedback synchrony to comparable degrees.

To explore the time course of the effect of muting GnRH drive on secretory regularity, LH and T ApEn and LH-T and T-LH cross-ApEn were computed during the arbitrary first, middle, and last 6 h of blood sampling. Ganirelix administration increased LH regularity in the first 6-h time interval and T regularity in the second 6-h interval (both *P* < 0.009). LH and T regularity returned to baseline values thereafter (third 6-h interval). Cross-ApEn of joint LH-T synchrony evolved in parallel with ApEn of T, whereas that for T-LH decreased in both the first and second 6-h intervals (*P* < 0.01).

Figure 2 illustrates the relationships between age and ApEn or cross-ApEn in the 18-h sampling periods associated with administration of 0.3 mg/m² ganirelix (Fig. 2A) and 1.0 mg/m² ganirelix (Fig. 2B). The effects of age on responses to the 0.3 mg/m² dose were modest (0.042 > *P* > 0.039). In contrast, GnRH receptor blockade with 1.0 mg/m² ganirelix strongly unmasked positive correlations (*P* ≤ 0.004) between age and each of T irregularity (higher ApEn), LH-T feedforward asynchrony, and T-LH feedback asynchrony (higher cross-ApEn values; Table 1). The positive sign of each correlation indicates that T orderliness, LH-T synchrony, and T-LH synchrony are attenuated in older men. Similar, but less conspicuous, age effects were detectable for T ApEn and T-LH cross-ApEn after the lowest (0.1 mg/m²) dose of ganirelix (Table 1). Exploratory time course analyses revealed that the disruptive effects of age on univariate orderliness and bivariate synchrony were most prominent during the first 6 h.

Figure 3 illustrates representative log-linear regressions of LH ApEn, T ApEn, forward LH-T cross-ApEn (bottom left), and reverse T-LH cross-ApEn (bottom right) on age after administration of 0.3 (A) or 1.0 (B) mg/m² ganirelix in 25 men of the indicated ages. Data reflect 18 h of sampling. Pearson’s correlation coefficients and corresponding *P* values are given.

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![Fig. 1](http://ajpendo.physiology.org/) Impact of ganirelix dose (0, 0.1, 0.3 and 1.0 mg/m²) on approximate entropy (ApEn) of luteinizing hormone (LH; top right) and testosterone (T; top left) and cross-ApEn of LH-T feedforward (bottom left) and T-LH feedback (bottom right) in 25 healthy men. Subjects underwent blood sampling every 10 min for 18 h to generate paired LH and T concentration profiles. Data are means ± SE. *P* values were determined by repeated-measures ANOVA. Means with unique (unshared) alphabetic superscripts differ significantly (*P* < 0.05) by post hoc comparisons.

![Fig. 2](http://ajpendo.physiology.org/) Regression of LH ApEn (top left), T ApEn (top right), forward LH-T cross-ApEn (bottom left), and reverse T-LH cross-ApEn (bottom right) on age after administration of 0.3 (A) or 1.0 (B) mg/m² ganirelix in 25 men of the indicated ages. Data reflect 18 h of sampling. Pearson’s correlation coefficients and corresponding *P* values are given.

![Fig. 3](http://ajpendo.physiology.org/) Each regression yielded a negative slope. The absolute value of any given slope provides an estimate of the fractional increment in regularity or joint regularity induced by a unit increase in ganirelix dose (sensitivity). To evaluate how age affects the sensitivity of LH ApEn,
orderliness of LH and T secretion (each action enhances the regularity of T secretion and the joint ANCOVA model revealed that COVA was employed to evaluate whether age, ganirelix dose, on age (Fig. 2).

age-related changes than direct regression of LH-T cross-ApEn

this regression model affords less statistical power in detecting

significant increases with age indicate progressive loss of ex-

minimally increases the regularity of LH (P = 0.009). Because higher ApEn and cross-ApEn denote greater irregularity, signif-

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pected ganirelix dose-dependent augmentation T regularity and

orderliness and forward LH-T and reverse T-LH synchrony in an antagonist dose- and time-dependent manner. Regression analyses showed that age markedly impaired enhancement of the regularity of T secretory patterns and the synchronicity of LH feedforward on T secretion and T feedback on LH secretion at the two higher doses of the GnRH antagonist. These outcomes strongly support the a priori postulate that age disrupts the quantifiable integrative capability of the male gonadal axis to adapt to controlled decrements in LH and T concentrations.

Twenty-four-hour and intensive overnight sampling protocols indicate that older men secrete LH and T more irregularly and jointly more asynchronously than young individuals (36, 48, 50, 68). The accompanying data replicate these findings over the first 6 h of observation (1800–2400), but unexpectedly not over the intervals comprising 2400–0600 or 0600–1200. The basis for apparent time segmentation of age-related contrasts in LH/T regularity is not known but could reflect subtle differences in statistical power (45) or include age-dependent effects on regularity of sleep cycles, circadian rhythmicity, and/or overnight fasting (2, 4, 63).

LH secretory bursts represent a relevant, but not necessarily exclusive, feedforward signal to the testis (9, 25, 34, 66). Conversely, T concentrations provide significant feedback to the hypothalamo-pituitary unit (69, 78, 82). Both linkages are

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T ApEn, LH-T, and T-LH cross-ApEn values to ganirelix dose, slope estimates were regressed linearly on age. The sensitivity of T ApEn and T-LH cross-ApEn to ganirelix dose increased significantly with age (both r = 0.51 and P = 0.009). Because higher ApEn and cross-ApEn denote greater irregularity, significant increases with age indicate progressive loss of expected ganirelix dose-dependent augmentation T regularity and LH-T synchrony with age. A nonsignificant similar trend was seen for forward LH-T synchrony (P = 0.10), indicating that this regression model affords less statistical power in detecting age-related changes than direct regression of LH-T cross-ApEn on age (Fig. 2).

As an independent statistical measurement, two-way ANCOVA was employed to evaluate whether age, ganirelix dose, and their interaction determine single-hormone regularity or bivhormonal synchrony over 18 h of sampling (Table 2). The ANCOVA model revealed that I) graded inhibition of GnRH action enhances the regularity of T secretion and the joint orderliness of LH and T secretion (each P < 10–4) and minimally increases the regularity of LH (P = 0.038); 2) age blunts the capability of increasing ganirelix doses to enhance T orderliness and forward LH-T and reverse T-LH synchrony (each P ≤ 0.0025); and 3) ganirelix dose is an independent determinant of LH and T ApEn and LH-T and T-LH cross-ApEn (all P < 10–4). Segmentation of ApEn and cross-ApEn analyses over successive arbitrary 6-h time windows indicated that age reduces secretory regularity most prominently during initial suppression (Table 2).

Figure 4 verifies that ganirelix dose (P < 10–4) but not age (P > 0.40) determines serum ganirelix concentrations. We did not study follicle-stimulating hormone because of its slow and minimal immediate suppression by GnRH receptor antagonists.

Table 1. Pearson correlation coefficients relating ApEn and cross-ApEn to age: impact of ganirelix dose and time after ganirelix injection

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Data are from 25 men. LH, luteinizing hormone; ApEn, approximate entropy; T, testosterone. NS, not significant. *0.01 < P < 0.05 and †P < 0.01.

DISCUSSION

The present investigation utilized an experimental strategy of escalating doses of a selective GnRH receptor antagonist to

enforce graded reductions in gonadotrope LH and thereby Leydig cell T secretion in 25 healthy men ages 20–72 yr. The resultant paired strata of LH and T output allowed quantification of the impact of age on the individual regularity of LH and T secretion patterns and the joint synchrony of LH-T feedforward and T-LH feedback coupling across distinct echelons of gonadal-axis outflow. In the cohort as a whole, stepwise muting of hypothalamic GnRH stimulation increased LH and T orderliness and forward LH-T and reverse T-LH coordination in an antagonist dose- and time-dependent manner. Regression analyses showed that age markedly impaired enhancement of the regularity of T secretory patterns and the synchronicity of LH feedforward on T secretion and T feedback on LH secretion at the two higher doses of the GnRH antagonist. These outcomes strongly support the a priori postulate that age disrupts the quantifiable integrative capability of the male gonadal axis to adapt to controlled decrements in LH and T concentrations.

Conventional linear methods of assessing two-hormone synchrony, such as cross-correlation or cross-spectral analyses, are complementary to cross-ApEn (48). Linear methods assume relatively fixed delays between the two time series (e.g., a uniform time delay for T responses to LH and vice versa). On the other hand, cross-ApEn provides a probabilistic estimate of relative pattern reproducibility that is independent of time delays between the paired signals (48, 49). Cross-correlation analyses also identified putative erosion of both LH-T feedforward and T-LH feedback (35, 70). The fact that two distinct but complementary methodologies predict impaired feedforward
and feedback regulation in aging men provides strong evidence for disruption of both pathways.

The precise neuroanatomic loci that contribute to aging-associated erosion of coordinate T-LH feedback signaling are

Table 2. Impact of age and time after ganirelix administration on ApEn and cross-ApEn

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Responses were observed in 25 men each studied after exposure to saline and 3 randomly ordered doses of ganirelix on separate days (100 subject-sessions of 18 h each). Data are \( P \) values determined by ANCOVA (see METHODS). NS denotes \( P > 0.05 \).

Fig. 3. Illustrative impact of ganirelix dose on the quantifiable regularity of LH and T secretion in a young (left) and older (right) volunteer of the indicated ages. Regularity measures include (from top to bottom) LH ApEn, T ApEn, LH-T cross-ApEn, and T-LH cross-ApEn.

Fig. 4. Age does not modify the dose-dependent increase in serum ganirelix concentrations in healthy men (\( n = 25 \)).
not known. Available data indicate that T and its metabolites exert inhibitory effects on the hypothalamus and pituitary gland (55, 62, 63, 69). The manner in which aging affects one or both sites of T action has not been elucidated (68, 75, 77). However, a recent GnRH dose-response study in healthy men indicated that aging decreases the regularity of acutely GnRH-stimulated LH secretion (71). Earlier biochemical studies in the male rodent suggested that senescence reduces hypothalamic-pituitary concentrations of the androgen receptor (13, 53). Whether or not androgen receptor expression also decreases in the older human, decreased concentrations of free (protein-unbound) and non-sex hormone-binding globulin-bound (bioavailable) T in aging may uncouple T-LH feedback synchrony (24). The last conjecture reflects the observation that pharmacological T deprivation induces markedly irregular LH secretion, even in young individuals (78).

Regression analyses showed that T ApEn, LH-T feedforward cross-ApEn, and T-LH feedback cross-ApEn all increased with age, denoting loss of individual T regularity and joint LH-T synchrony. At the highest dose of the GnRH-receptor antagonist tested, r² values were 0.43 to 0.49, thus indicating that age could explain ~45% of the intersubject variability in these distinct measures. Exploratory assessment of the time dependence of age contrasts disclosed prominent differences during the suppressive phase of ganirelix action (Table 1). Such data could indicate that aging especially impacts rapid adaptations of the male gonadal axis. If valid, this inference may have clinical relevance to understanding blunted LH-T responses to acute stressors in aging men.

In summary, an experimental paradigm comprising randomly ordered, separate-day administration of saline and varying doses of a selective GnRH receptor antagonist delineates prominent age-related loss of ensemble gonadal axis adaptability to partial silencing of a single agonistic pathway. Prominent effects of aging included disruption of T regularity, LH-T feedforward coordination, and T-LH feedback synchrony. Longitudinal studies will be required to establish the precise age dependencies of these inferred defects.

GRANTS

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