CLINICAL STUDY

Two-week pulsatile gonadotropin releasing hormone infusion unmasks dual (hypothalamic and Leydig cell) defects in the healthy aging male gonadotropic axis

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Abstract

Objective: To examine the possibility that lower serum bioavailable testosterone concentrations, without increased LH release, in healthy older men, reflects hypothalamic GnRH deficiency.

Design: We used a randomized, double-blind, placebo-controlled design.

Methods: We treated each of five young (ages 20–34 years) and five older (ages 60–78 years) men with 2 weeks of randomized infusions of saline or pulsatile GnRH (100 ng/kg i.v. every 90 min).

Results: At baseline (saline infusion), older men had more LH pulses (young compared with old, 10 ± 0.6 compared with 15 ± 1 , $P = 0.0026$) per 24 h, reduced fractional LH pulse amplitude ($219 \pm 17\%$ compared with $167 \pm 40\%$, $P = 0.0376$), and more disorderly hormone release as judged by approximate entropy (ApEn) (LH, $P \leq 0.0001$; testosterone, $P \leq 0.0047$). In response to pulsatile i.v. GnRH infusions, serum 24-h LH concentrations (measured by immunoradiometric assay (IRMA)), increased equivalently in young and older men (to 7.3 ± 1.2 and 7.2 ± 1.8 IU/l respectively). GnRH treatment also normalized LH pulse frequency and amplitude, ApEn, and plasma biologically active LH (pooled) concentrations. In contrast, 24-h testosterone concentrations failed to increase equivalently in older men (young compared with old, 869 ± 88 compared with 517 ± 38 ng/dl, $P = 0.0061$), reflecting lower testosterone peak maxima (995 ± 108 compared with 583 ± 48 ng/dl, $P = 0.0083$) and interpeak nadirs (750 ± 87 compared with 427 ± 26 ng/dl, $P = 0.0073$).

Conclusions: We have demonstrated that, in older men, successful reconstitution of 24-h pituitary (bioactive) LH output and pulsatile (IRMA) LH release patterns could be achieved by a fixed exogenous GnRH pulse signal, thereby implicating altered endogenous hypothalamic GnRH release in the relative hypogonadotropism of aging. The failure of testosterone concentrations to increase concomitantly points to a simultaneous Leydig cell defect. We conclude that aging in men is marked by a dual defect in the central nervous system–pituitary–Leydig cell axis.

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Introduction

Multiple regulatory factors (e.g., sex steroids, polysynaptic neuronal inputs) coordinate hypothalamic secretion of bursts of gonadotropin-releasing hormone (GnRH) in women and men (1). Pulsatile release of GnRH into the hypothalamic–pituitary portal microcirculation in turn stimulates (feedforward) episodes of pituitary secretion of luteinizing hormone (LH). Intermittent secretion of LH into the systemic circulation correspondingly drives pulsatile gonadal production of testosterone in men (2). Biologically active testosterone in the plasma completes the feedback control loop, via time-lagged inhibition of GnRH and LH secretion (3, 4).

The negative impact of aging on the male hypothalamic–pituitary–gonadal axis is important, because diminished sex-steroid secretion in men is accompanied by decreased muscle mass and strength, reduced bone mineral density and greater risk of hip fracture, loss of sexual interest, increased risk of coronary artery disease, and impaired psychological well-being and spatial cognition (5–9). Mechanistically, attenuation of gonadal testosterone secretion could be caused by disrupted feedforward or feedback interactions among the key control sites within the male reproductive axis, viz. the hypothalamus, pituitary gland, or Leydig cells. Based on available indirect clinical data (10–12), we postulated that diminished hypothalamic GnRH secretion in healthy older men

gives rise mechanistically to the reduced pituitary LH and Leydig cell testosterone secretion.

To test the hypothesis of diminished brain GnRH secretion in older men, we administered synthetic GnRH or saline, in randomized order, as pulsatile i.v. infusions for 2 weeks, to young and to older individuals at a weight-adjusted GnRH dose that normalizes LH secretion in hypogonadotropic men (13). On day 14 of GnRH or saline treatment, we compared individual and joint LH and testosterone release as quantitated by validated pulse and entropy analyses in older and young individuals.

Methods

This study was approved by the University of Virginia Human Investigation Committee. We studied healthy young ($n = 5$, age 20–34 years) and older ($n = 5$, age 60–78 years) men, who had no acute or chronic illness, ingested no drugs or medications, were non-smokers, were within 20% of ideal body weight, and had not undertaken any transmeridian travel in the past 2 weeks.

After giving written informed consent, participants were connected to a pocket-sized GnRH infusion pump (Ferring Laboratories) with an i.v. catheter placed in a forearm vein. Volunteers then received 2 weeks of either saline or GnRH (administered as 100 ng/kg i.v. bolus injections every 90 min). The study design was a randomized, double-blind, cross-over trial of saline compared with GnRH. On day 14 of GnRH or saline infusions, the men spent 36 h in the General Clinical Research Center. The first 12 h overnight were to acclimate the individual to the setting. During the next 24 h (0800 h until 0800 h the following morning), while the pulsatile GnRH or saline infusion continued, volunteers underwent venous sampling every 10 min (145 samples). Blood samples were allowed to clot at room temperature and the sera were frozen at -20°C for later assay of LH and testosterone.

We performed serum LH assays in duplicate via a fully automated robotics system, using a two-site monoclonal immunoradiometric assay (IRMA, Nichols Laboratory, San Juan Capistrano, CA, USA). The sensitivity of the LH IRMA was 0.10 IU/l (First International Reference Preparations). The inter- and intra-assay coefficients of variation were $<5.5\%$. Serum total testosterone measurements were performed in duplicate on each sample, using an RIA (Diagnostic Products, Los Angeles, CA, USA) with an assay sensitivity of 20 ng/dl and inter- and intra-assay coefficients of variation of $<10\%$. Bioavailable testosterone, biologically active LH, and inhibin B concentrations in 24-h serum pools were assayed exactly as described previously (14–16). FSH, prolactin, IGF-I, DHEA and estradiol were also measured.

For pulse analysis of the LH and testosterone time series, we used Cluster analysis, as a model-free discrete peak detection algorithm, as described previously (17). Approximate entropy (ApEn) was applied to quantitate

the relative disorderliness of the LH (or testosterone) release process, as previously reported (18).

For comparisons of the effect of GnRH or saline within either the young or older cohorts, we used the Wilcoxon two-tailed matched-pairs signed-ranks test. For comparisons between the young and older men, we used the unpaired Mann–Whitney U (rank sum) test. $P < 0.05$ was construed as statistically significant.

Results

Illustrative serum concentration time series for LH (IRMA) and total testosterone (RIA) are presented in Figs 1 and 2. During the control period (saline), young and older subjects had no significant differences in their mean 24-h serum LH concentration by IRMA (young compared with older, 3.2 ± 0.56 compared with 3.2 ± 0.40 IU/l) or in total testosterone concentrations (young compared with older, 460 ± 39.8 compared with 397 ± 35.0 ng/dl). However, control (pre-GnRH) bioactive LH concentrations (young compared with older, 28 ± 13 compared with 15 ± 2.7 IU/l) and bioavailable testosterone concentrations tended to be lower in older men (young compared with older, 383 ± 13 compared with 181 ± 16 ng/dl). Findings for other hormones (FSH, prolactin, and dihydroepiandrosterone) are outlined in Table 1.

By Cluster analysis, older men had more frequent peaks of serum LH (young compared with older, 10 ± 0.6 compared with 15 ± 1 , $P = 0.0026$) per 24 h, and reduced serum LH peak amplitudes (young compared with older, $219 \pm 17\%$ compared with $167 \pm 40\%$, $P = 0.0376$).

Approximate entropy (ApEn) of 24-h LH release in the control session was markedly increased, indicating greater disorderliness, in the older men (young compared with older, 0.699 ± 0.076 compared with 1.523 ± 0.063 , $P = 0.0001$), as was the ApEn for 24-h testosterone release (young compared with older, 1.399 ± 0.080 compared with 1.710 ± 0.009 , $P = 0.0047$). Cross-ApEn of joint LH–testosterone synchrony over 24-h was also higher in the older individuals (young compared with older, 1.351 ± 0.119 compared with 2.010 ± 0.050 , $P = 0.0009$).

After 2 weeks of pulsatile i.v. GnRH infusions, mean serum 24-h LH concentrations increased equivalently in young and older men (by IRMA, to 7.3 ± 1.2 compared with 7.2 ± 1.8 IU/l). Bioactive LH also increased statistically similarly in both age groups (young compared with older, 57 ± 13 compared with 31 ± 6.6 IU/l, $P = \text{NS}$). Cluster-identified LH pulse frequency and amplitude, and ApEn, became statistically identical in the young and older men (Table 2). In contrast, serum (24-h mean) total testosterone concentrations with GnRH treatment increased less in older men (Fig. 3), because of lower 24-h serum testosterone peak areas, peak maxima, and interpeak nadirs (Table 2). In addition, the relationship between incremental serum

Figure 1 Illustrative 24-h serum LH concentration time series of three young and three older men, obtained while they were receiving pulsatile i.v. saline (control) or GnRH infusion every 90 min for 14 days. Each data point with its associated error bar represents the mean ($\pm$ dose-dependent s.d.) of the serum LH concentration determined by IRMA in each 10-min blood sample collected over 24 h. Note the more disorderly appearance of the (24-h) LH time series during the control period in the older men.

Figure 2 Serum total testosterone concentration time series of three representative young and older men sampled over 24 h while they were receiving saline (control) or GnRH infusions. The data points in each time series represent the mean (of duplicate) serum testosterone concentrations for each 10-min sample with its associated error bar. Note the lower serum testosterone concentrations in the older men during the GnRH infusion period.

Table 1 Mean ($\pm$ S.E.M.) hormone concentrations during control or GnRH infusions in young ($n = 5$) and older $n = 5$) men.

Hormone	Control period			GnRH period		
	Young	Older	<i>P</i> value	Young	Older	<i>P</i> value
FSH (IU/l)	4.8 $\pm$ 2.1	4.0 $\pm$ 1.1	NS	5.4 $\pm$ 1.4	6.4 $\pm$ 2.5	NS
Prolactin (μ g/dl)	8.7 $\pm$ 0.89	4.9 $\pm$ 0.62	0.0074	13.7 $\pm$ 1.7	6.3 $\pm$ 1.2	0.0067
IGF-I (μ g/l)	249 $\pm$ 29.1	123 $\pm$ 33.4	0.022	325 $\pm$ 50	137 $\pm$ 31	0.013
DHEA (μ g/dl)	251 $\pm$ 20.5	118 $\pm$ 36	0.013	306 $\pm$ 35.6	128 $\pm$ 48.3	0.018
Inhibin B concn (pg/ml)	78 $\pm$ 6.4	77 $\pm$ 3.4	NS	69 $\pm$ 3.0	65 $\pm$ 2.4	NS

concn, concentration.

LH concentrations and increased testosterone concentrations was markedly blunted in the older men (Fig. 4). Bioavailable testosterone also failed to increase in older men receiving GnRH pump treatment ($P = 0.0026$). Specifically, the values in young men increased to 669 ± 104 ng/dl compared with 268 ± 31 ng/dl in older volunteers (Fig. 5). Confirming Leydig cell failure in the older men, serum estradiol concentration also did not increase in the older men while they were receiving GnRH (Fig. 6). Serum prolactin and insulin-like growth factor-I (IGF-I) concentrations similarly failed to increase significantly in the older men (Figs 7, 8).

There was also a markedly blunted relationship between the increase in serum total testosterone concentrations and changes in serum IGF-I concentrations in the older men (Fig. 9). In particular, as the serum total testosterone concentration increased during GnRH infusion in young men, so did the serum IGF-I concentration. In contrast, there was no such positive association in the older men. Neither age group showed greater (pooled 24-h) serum inhibin B concentrations during GnRH infusions (Table 1).

Discussion

The present clinical investigation is, to our knowledge, the first *in vivo* assessment of the human pituitary–Leydig cell axis in healthy older men using a short-term (2-week) pulsatile i.v. GnRH ‘clamp’ technique to

impose an experimentally uniform (exogenous) GnRH pulse stimulus. This clinical strategy serves to isolate the pituitary–Leydig cell unit under an unvarying (extrinsically controlled) hypothalamic peptide releasing-factor drive, thus eliminating potential confounding influences arising from the unequal hypothalamic GnRH input in young and older individuals. In this experimental paradigm, we established that aging is not associated with measurably impaired gonadotrope secretion of either biologically active or immunologically reactive LH, when young and older men are given equivalent pulsatile GnRH stimulation. In both young and older men, 2 weeks of i.v. pulsatile GnRH treatment elicited quantitatively identical 24-h serum LH concentration profiles, with statistically indistinguishable pulsatile LH output (assessed by Cluster analysis) and orderliness of LH release (quantitated by approximate entropy). The absence of any apparent age-related deficit in exogenous GnRH-driven immunoreactive (or 24-h pooled bioactive) LH secretion speaks for a preserved pituitary gonadotrope secretory capacity and responsiveness in older men.

Previous investigators have studied the endogenously regulated gonadotrope–Leydig cell axis in older men, but have published conflicting results. Two recent studies evaluating bioactive or immunoreactive LH release after graded submaximal bolus doses of GnRH suggested increased age-related (maximal) LH secretion (19, 20). Other reports utilizing a single high(er) dose of GnRH

Table 2 Pulsatile LH and testosterone release during control or GnRH infusions in young ($n = 5$) and older ($n = 5$) men.

	Control period			GnRH period		
	Young	Older	<i>P</i> value	Young	Older	<i>P</i> value
LH						
Pulse frequency (1/24 h)	10 $\pm$ 0.5*	15 $\pm$ 1.0	0.0026	15 $\pm$ 0.7	13 $\pm$ 0.9	NS
Pulse amplitude (%)	219 $\pm$ 17	167 $\pm$ 40	0.0376	168 $\pm$ 11	153 $\pm$ 10	NS
ApEn	0.699 $\pm$ 0.076	1.523 $\pm$ 0.063	0.0001	1.296 $\pm$ 0.067	1.387 $\pm$ 0.173	NS
Testosterone						
Pulse area (ng/dl $\times$ min)	6239 $\pm$ 1247	3633 $\pm$ 608	NS	9105 $\pm$ 609	5478 $\pm$ 1324	0.00375
Pulse height (ng/dl)	508 $\pm$ 33.5	447 $\pm$ 39.4	NS	995 $\pm$ 108	583 $\pm$ 48.2	0.0083
Interpeak nadir (ng/dl)	380 $\pm$ 28.2	331 $\pm$ 25.0	NS	750 $\pm$ 86.7	427 $\pm$ 26	0.0073
ApEn	1.399 $\pm$ 0.080	1.710 $\pm$ 0.009	0.0047	1.595 $\pm$ 0.069	1.671 $\pm$ 0.077	NS

ApEn, approximate entropy; higher values denote more disorderly hormone release.

Figure 3 Comparison of the increments (GnRH-treated minus control) in mean (24-hr) serum (total) testosterone concentrations in five individual young and five individual older men while they were receiving a 2-week pulsatile i.v. GnRH infusion. Note the much smaller increment in mean serum testosterone concentrations in the older men. Values are the mean $\pm$ S.E.M., and the *P* value denotes the non-parametric group (young compared with older) comparison.

Figure 4 Comparison of the relative increase (increment) in the mean (24-h) serum testosterone concentration for a given increment in the mean (24-h) serum LH concentration in young and older men. Note the marked blunting of the LH-driven testosterone increment in the older men. Equation and *R* value are for the linear regression (dotted line) of LH increase on testosterone increment in young men (*P* = NS in older men, (solid line)).

Figure 5 Serum (pooled 24-h) bioavailable testosterone concentrations in young and older men during control (saline) or pulsatile GnRH infusions. The *P* value was determined by ANOVA; different superscripts denote significantly different treatment means ($\pm$ S.E.M.).

Figure 6 Serum (pooled 24-h) estradiol concentrations in young and older men during control (saline) or pulsatile GnRH infusions. The *P* value was determined by ANOVA; different superscripts denote significantly different treatment means ($\pm$ S.E.M.). Note the attenuated GnRH-driven increase in the serum estradiol concentration in the older men.

Figure 7 Serum (pooled 24-h) prolactin concentrations in young and older men during control (saline) or pulsatile GnRH infusions. The *P* value was determined by ANOVA; different superscripts denote significantly different treatment means ($\pm$ S.E.M.). Note the attenuated GnRH-driven increase in the serum prolactin concentration in the older men.

Figure 8 Serum (pooled 24-h) IGF-I concentrations in young and older men during control (saline) or pulsatile GnRH infusions. The *P* value was determined by ANOVA; different superscripts denote significantly different treatment means ($\pm$ S.E.M.). Note the attenuated GnRH-driven increase in the serum IGF-I concentration in the older men.

Figure 9 Comparison of the relative increase in the serum (24-h pooled) IGF-I concentration for a given increment in the (24-h mean) serum total testosterone concentration in young (dotted line) and older men (solid line). Note the absence of a positive association between serum testosterone and IGF-I concentrations in older individuals.

inferred an age-associated impairment of gonadotrope responsiveness (21–23). However, none of these studies attempted to mimic normal GnRH pulse physiology over a multi-day period, for example by evaluating the pituitary–Leydig unit responses to >24 h of near-physiological i.v. pulses of GnRH in young and in older men. Here, we imposed a 2-week randomized intervention of pulsatile saline or GnRH infusions to achieve uniform and near-physiological hypothalamic decapeptide drive to gonadotrope cells in young and older men. Our finding of statistically comparable entropy of 24-h LH release and LH pulse frequencies and amplitudes under fixed exogenous GnRH stimulation (but not basally under endogenous GnRH drive) in young and older men strongly suggests that alterations in endogenous GnRH release in older men underlie the spontaneous low-amplitude, higher frequency, and more disorderly LH secretion patterns that mark this age group. Indeed, most studies in older men show a relative loss of high-amplitude LH pulses, with or without an evident replacement by higher-frequency low-amplitude peaks (24–26). Our inference that endogenous GnRH release is attenuated in aging men is consistent with extant animal studies, which have demonstrated diminished brain estrogen-receptor expression in aged male rats (27), ultrastructural changes to GnRH neurons (28), diminished hypothalamic neuronal oscillations (29), hypothalamic-transplant reversible impotence (30), and attenuated GnRH release in response to excitatory amino acids in older rodents (31).

Despite experimentally induced statistical equalization of pulsatile and entropic LH release patterns and amounts in young and older men, 24-h serum testosterone concentrations and release dynamics remained abnormal in older compared with young adults. The mean (24-h) serum total and bioavailable testosterone concentrations in older men remained diminished during GnRH treatment, as did the maximal amplitude of the

individual serum testosterone peaks, their areas, and the interpeak nadirs. These data suggest reduced LH bioactivity, impaired Leydig cell perfusion, a Leydig cell secretory defect, or a combination of these disruptions in aging men. Reduced LH bioactivity on GnRH pump treatment was excluded by *in vitro* Leydig cell bioassay of 24-h (pooled) serum LH concentrations. However, the last two mentioned putative explanations would concur with prior studies demonstrating decreased testicular perfusion, fewer Leydig cells, diminished responses to human chorionic gonadotropin, and variable steroidogenic defects in older men (32, 33). Whereas some studies have suggested decreased basal LH bioactivity/immunoreactivity ratios in older men to explain the discordance between LH and testosterone levels (34, 35), another report refuted a hypothesis of a gross qualitative difference in the LH isoform profiles in young and older males, but did not eliminate the possibility of reduced LH bioactivity (36). In other investigations, basal LH bioactivity or immunofluorimetric activity was normal in healthy older men (37, 38), and only reduced in illness or disease (24). Moreover, LH bioactivity in older men increased normally after interruption of androgen-negative feedback (39), but not during antiestrogen treatment (40). Given impaired gonadal testosterone biosynthesis in aging men and rodents (2, 32, 41, 42), we favor the notion that a steroidogenic defect in Leydig cells accounts for reduced pulsatile testosterone release in older men, despite a GnRH-stimulated (and biologically active) LH pulse signal indistinguishable from that achieved in similarly treated young adults. Any putative defect at the Sertoli cell level in aging men could not be evaluated here, given the failure of inhibin B concentrations (of Sertoli cell origin) to increase during GnRH treatment in either age group. In the present study, basal concentrations of inhibin B were not lower in older men, unlike data from earlier alpha-subunit directed assays (43, 44).

We further noted a positive association between the GnRH–LH-stimulated serum testosterone concentration and the serum IGF-I concentration in the younger men, but a loss of this association in the older men. This observation suggests at least one biological implication at the tissue level of limited testosterone secretory reserve in older individuals.

In summary, the achievement of quantitatively and qualitatively equivalent frequency, amplitude and orderliness of the 24-h serum LH release profiles after 2 weeks of near-physiological pulsatile i.v. GnRH infusions in older men points to GnRH-dependent (hypothalamic) alterations underlying the qualitative disruptions in basal LH secretion otherwise evident in the aging male gonadotropic axis. Our analyses also disclose reduced LH-driven testosterone release in healthy older men, thus indicating a concomitant defect in the Leydig cell of older individuals. In these respects, we suggest that the aging human male and female gonadotropic axes behave mechanistically similarly, with joint hypothalamic–gonadal alterations (45).

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