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Secretagogues govern GH secretory-burst waveform and mass in healthy eugonadal and short-term hypogonadal men

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Abstract

Background—GH pulses are putatively initiated by hypothalamic GH-releasing hormone (GHRH), amplified by GH-releasing peptide (GHRP), and inhibited by somatostatin (SS).

Objective—To ascertain how secretagogues control the waveform (time evolution of release rates) as well as the mass of secretory bursts.

Design—We quantified the shape of GH secretory bursts evoked by continuous combined i.v. infusion of maximally effective doses of GHRH and GHRP-2, and by bolus injection of each peptide after delivering L-arginine to restrain hypothalamic SS release in 12 healthy young men.

Methods—A mathematically verified and experimentally validated variable-waveform deconvolution model was applied to intensively sampled GH time series.

Results—The secretory-burst mode (time from burst onset to maximal secretion) was 19±0.69 min during saline infusion, and fell to a) 10.4±3.0 min during constant dual stimulation with GHRH/GHRP-2 ($P<0.01$), b) 14.6±1.8 min after L-arginine/GHRH ($P<0.025$), and c) 15.0±1.0 min after L-arginine/GHRH ($P<0.01$). Secretagogues augmented the mass of GH secreted in pulses by 44-, 42-, and 16-fold respectively, over saline (2.2±0.81 μ/l per h; $P<0.001$ for each). Pulse number and variability were unaffected. Applying the same methodology to ten other young men with acute leuprolide-induced hypogonadism yielded comparable waveform and mass estimates.

Conclusion—The present analyses in men demonstrate that peptidyl secretagogues modulate not only the magnitude but also the time course of the GH-release process *in vivo* independently of the short-term sex-steroid milieu.

Introduction

Growth hormone (GH) secretion is principally (>85%) pulsatile, as monitored by direct sampling of hypothalamo-pituitary portal blood in the monkey, sheep, pig, and rat; peripheral measurements in the humans; and *in vitro* perifusion of pituitary cells (1,2). In principle, physiological control could be exerted on the number (frequency), size (mass), and/or shape (time evolution or waveform) of GH secretory bursts. Little is known about *in vivo* regulation of the waveform of the hormone secretory process. A major obstacle to such investigations has been the lack of a flexible analytical model that embodies both an initial rapid increase in secretion (due to exocytosis of membrane-associated secretory vesicles) and a delay in

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Declaration of interest
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secretion (due to *de novo* hormone synthesis and granule recruitment) (3-6). A new analytical formalism was introduced recently to approach this basic problem. The methodology exploits a three-parameter model of variably asymmetric secretory-burst waveforms that characterize pulsatile secretion of thyroid-stimulating hormone, adreno-corticotropin, luteinizing hormone (LH), and GH (in women) (7-9). The mathematical strategy comprises iterative estimation of all secretion and elimination parameters simultaneously, including the unknown waveform and mass of secretory bursts and underlying non-pulsatile (basal) secretion, conditional on *a priori* identification of candidate sets of pulse times via a recently published pulse-detection procedure (10). The optimal pulse-time set is then selected using the Bayesian information criterion.

The variable-waveform deconvolution model was validated experimentally using frequently sampled pituitary hormone time series in the horse, sheep, and humans, and verified mathematically by direct statistical proof (8-11). A novel inference in studies to date is that both the size and shape of secretory bursts may be regulated by secretagogue type, hormonal milieu, negative feedback, age and time of day (7,11,12).

In postmenopausal women, estrogen appears to extend the duration of GH secretory bursts, whereas peptidyl secretagogues may reverse this effect (13,14). Such observations suggest that sex steroids and secretagogues may exert opposing effects on mechanisms that control the GH release process. To examine this proposition further, the present analyses were conducted in the androgen-rich estrogen-poor milieu of healthy young men and, for comparison, the combined androgen/estrogen-depleted milieu of gonadal axis downregulated young men. The goal was to elucidate individual and interactive effects of GH-releasing hormone (GHRH) and GH-releasing peptide (GHRP-2) on the shape and size of GH secretory events in distinct sex-steroid milieus.

Subjects and methods

Subjects

Volunteers provided written informed consent, which was approved by the Mayo Institutional Review Board and reviewed by the US Food and Drug Administration under an investigator-initiated new drug number. Exclusion criteria were exposure to psychotropic or neuroactive drugs within five biological half-lives; body mass index (BMI)>32.5 kg/m^2; anemia (hemoglobin <12.8%); drug or alcohol abuse, psychosis, depression, mania, or recurrent stress; acute or chronic organ-system disease; use of sex hormones, anabolic steroids, or glucocorticoids; endocrinopathy, other than primary thyroidal failure receiving replacement; nightshift work (within 2 weeks) or recent transmeridian travel (exceeding three time zones within 7 days of admission); acute weight change (loss or gain of >2 kg in 6 weeks); allergy to administered peptides; and unwillingness to provide written informed consent. Each subject had an unremarkable medical history and physical examination and normal screening laboratory tests of hepatic, renal, endocrine, metabolic, and hematologic function. The men reported normal sexual development and function.

Protocol

Ten healthy young men received two consecutive injections of depot leuprolide acetate (3.75 mg i.m. 3 weeks apart) to deplete systemic testosterone and estradiol (E_2) concentrations. Secretagogue infusions were scheduled 10-18 days after the second dose of leuprolide. Twelve other young men were not given leuprolide. Each subject was studied three times in the Clinical-Translational Research Unit (CRU). Admissions were scheduled at least 48 h apart on separate mornings after a standardized overnight fast (below). The study design was parallel-cohort, double-blind, and prospectively randomized. Subjects were admitted to the CRU before

1700 h and stayed overnight, or arrived by 0630 h on the day of infusion. To limit nutritional confounds, a constant meal (vegetarian or non-vegetarian) was given to ingest at home or in the CRU at 1800 h the night before the study comprising 8 kcal/kg distributed as 50% carbohydrate, 20% protein, and 30% fat. Volunteers then remained fasting, alcohol-abstinent, and caffeine-free overnight until the end of the infusion the next day.

In the CRU, i.v. catheters were placed in contralateral forearm veins at 0700 h to allow simultaneous infusion of saline or secretagogues. Blood sampling was done every 10 min for 6 h beginning at 0800 h during saline infusion, and separately every 10 min for 3.5 h in relation to defined secretagogue infusions. The blood was obtained during a 1 h baseline interval (0800-0900 h) and subsequent 2.5 h (0900-1130 h) secretagogue infusion interval (Fig. 1). I.v. infusions comprised: i) combined GHRH and GHRP-2 delivery both at a constant rate of 1 μ/kg per h; ii) L-arginine 30 g over 30 min, followed immediately by 1 μ/kg bolus GHRH (GEREF; Serono, Norwalk, MA, USA); and iii) L-arginine as in (ii) followed by 3 μ/kg bolus GHRP-2. These doses of L-arginine and peptides are maximally stimulatory in adults (15-17). L-arginine was employed to antagonize GH autofeedback-induced somatostatin outflow (18,19).

The blood was also withdrawn at 0800 h for later assay of serum E_2, testosterone, LH, and insulin-like growth factor-1 (IGF1) concentrations. Lunch was provided at noon before discharge from the CRU.

Hormone assays

Plasma GH concentrations were determined in duplicate by automated ultrasensitive double-monoclonal immunoenzymatic, magnetic particle-capture chemiluminescence assay using 22 kDa recombinant human GH as assay standard (Sanofi Diagnostics Pasteur Access, Chaska, MN, USA) (13). Sensitivity is 0.010 μ/l (defined as 3 S.D.S above the zero-dose tube). Inter-assay coefficients of variation (CVs) were 7.9 and 6.3% at GH concentrations of 3.4 and 12 μ/l respectively. Intra-assay CVs were 4.9% at 1.1 μ/l and 4.5% at 20 μ/l. No values fell less than 0.020 μ/l. Cross-reactivity with 20 kDa GH is less than 5% on a molar basis. Testosterone concentrations were quantitated by automated competitive chemiluminescent immunoassay (ACS Corning, Bayer). Mean intra- and inter-assay CVs were 6.8 and 8.3%, with an assay sensitivity of 8 ng/dl (multiply by 0.0347 for nmol/l) (20).E_2 was measured by double-antibody RIA (Diagnostic Products Corp., Los Angeles, CA, USA). Intra-assay CVs are 18.3% at 3.6 pg/ml, 3.8% at 40 pg/ml, and 7.2% at 297 pg/ml. Inter-assay CVs are 8.1, 4.7, and 4.9% at 16.0, 31, and 119 pg/ml respectively (multiply by 3.47 for pmol/l). Insulin-like growth factor binding protein (IGFBP)1, IGFBP3, and total IGF1 concentrations were measured by IRMA (Diagnostic Systems Laboratories, Webster, TX, USA) (20,21). Inter-assay CVs for IGF1 were 9% at 64 μ/l and 6.2% at 157 μ/l. Intra-assay CVs were 3.4% at 9.4, 3% at 55, and 1.5% at 264 μ/l.

Statistical analysis

An unpaired Student's *t*-test was used to compare baseline (unstimulated) hormone concentrations in the two groups. Two-way ANCOVA was applied to assess pulsatile GH secretion in a two-factor (eu- or hypogonadal (HYPO) status) by three-factor (three secretagogues) design, in which saline responses served as the covariate.

Data are presented as the mean±S.E.M. Experimentwise $P<0.05$ was construed as statistically significant.

Analytical methods

Earlier deconvolution methods in some cases yield non-unique estimates of basal and pulsatile hormone secretion and elimination rates (22). To address this technical impasse, basal and

pulsatile GH secretion rates were estimated simultaneously using a new maximum-likelihood deconvolution methodology presented in (7,10,11). The basic assumptions are that a) peaks in concentrations reflect underlying time-delimited secretory bursts; b) the burst waveform (time course of instantaneous release) is approximated by a flexible three-parameter generalized gamma probability density; c) combined diffusion, advection, and irreversible elimination are represented via biexponential kinetics; and d) simultaneous parameter estimation is statistically conditioned on *a priori* estimates of sets of candidate pulse onset times obtained by an incremental (selective) smoothing algorithm (Fig. 2). The optimal pulse-set model is then selected on probabilistic grounds using the Bayesian information criterion (BIC) (23).

A modification of the general model was implemented, wherein the principal analytical outcomes were cohort-specific estimates of basal and pulsatile GH secretion during saline infusion (μ/l per h); the summed mass of GH secreted in bursts after stimulation with secretagogues (μ/l per h); and the reconstructed shape of GH secretory bursts, defined by the modal time in minutes to attain maximal secretion after the objectively marked burst onset. The elimination model was a biexponential function with rate constants corresponding to rapid and slow GH half-lives of 3.5 and 20.8 min, apportioned as 37% rapid and 63% slow disappearance (24). Interpulse-interval lengths were modeled as a two-parameter Weibull renewal process (10). Unlike a one-parameter Poisson distribution that defines the CV ($_{S.D.}$/mean×100%) of interpulse intervals as a fixed value of 100%, the Weibull density includes an additional term (γ) that allows for lesser variability. In particular, interpulse-interval CV values are less than 100% for γ>1.0 independently of the probabilistic mean frequency (λ). When γ=1.0, the Weibull and Poisson processes are identical.

Results

Eugonadal (EU) and HYPO men were of similar age (22.7±0.71 and 22.8±1.3 years) and BMI (26±0.92 and 25±0.98 kg/m^2; mean±$_{S.E.M}$). On the days of peptide infusions, HYPO subjects had 10-fold lower serum total testosterone concentrations (P<0.001), 3-fold lower E_2 concentrations (P<0.001), and 2.2-fold lower LH concentrations (P=0.002) than EU subjects (Table 1). IGF1 concentrations (μ/l) were not different. Therefore, the leuprolide regimen enforced sex-steroid depletion without altering IGF1 concentrations.

GH concentration profiles in three EU and three HYPO men are depicted in Fig. 3. Data are 10 min time series collected during infusion of saline for 1 h followed by each of the three secretagogue pairs, GHRH/GHRP, L-arginine/GHRP-2, and L-arginine/GHRH. Deconvolution-estimated curves are projected over the measured GH time series to illustrate model performance.

During saline infusion, pulsatile GH secretion averaged 2.24±0.081 EU and 1.80±0.54 HYPO μ/l per h (P=NS). Statistical comparisons of stimulated pulsatile GH secretion revealed no differences between EU and HYPO cohorts. Data from the combined cohorts (N=22) showed a mean-weighted 54-fold effect of GHRH/GHRP-2 (P<0.001), 47-fold effect of L-arginine/GHRP-2 (P<0.001), and 20-fold effect of L-arginine/GHRH (P<0.01) over saline. The responses to GHRH/GHRP-2 and L-arginine/GHRP-2 both exceeded that of L-arginine/GHRH (P<0.01) but did not differ from each other (P>0.10). The inset graph in Fig. 4 shows that fasting basal (non-pulsatile) GH secretion (μ/l per h) was 2.5-fold higher in EU (0.30±0.078) than HYPO (0.12±0.039) men (P<0.025), and accounted for respectively 11.8 and 6.25% of total GH secretion.

Figure 5 depicts cohort-specific GH secretory-burst waveform plots. By visual inspection, secretagogues shortened the time latency between burst onset and maximal secretion, which was quantified formally by the waveform mode. Figure 6 summarizes analytically determined

modes of unstimulated (saline) and dual secretagogue-stimulated GH secretory bursts for each of the eight study conditions. On the saline day, the mode (±S.E.M.) was 19.2±0.69 min in EU and 21.2±1.33 min in HYPO subjects ($P>0.10$). Statistical comparisons indicated that a) stimulation with secretagogue pairs significantly reduced the time latency to maximal GH secretion ($P<0.01$ for the three secretagogue infusions considered together compared with saline infusion); b) the three secretagogue pairs were similar in their burst-abbreviating effects; c) the median effect expressed as a percentage reduction was 43% (absolute range 22-79%); and d) GH secretory-burst waveform responses in EU and HYPO subjects did not differ, indicating robustness of the secretory process to marked short-term sex-steroid depletion.

The precision of estimating the modes from the present data (expressed as S.E.M./mode×100%) was comparable among secretagogues, except for L-arginine/GHRH in HYPO (46%) and GHRH/GHRP-2 in HYPO (179%). These two values were significantly above the median (8.7%) and range (3.5-29%) for the other six groups, viz., saline (EU and HYPO 3.5 and 6.5%), L-arginine/GHRP-2 (12.7 and 15.2%), GHRH-GHRP-2 (EU 29%), and L-arginine/GHRH (EU 6.7%). The data raise the possibility that the HYPO state augments physiological variability in the shape of GH secretory bursts.

GH interpulse intervals (min) were modeled as a two-parameter Weibull renewal process or probability distribution (Fig. 7). Mean (±S.D.) interpulse intervals were 53±45 in EU and 44±31 in HYPO individuals, corresponding to GH pulse frequencies (λ, number per 24 h) of 30 and 26 respectively ($P>0.10$). The regularity term, γ, in the Weibull process was estimated as 1.4 (unitless) and 1.2 in EU and HYPO subjects respectively, which correspond to interpulse-interval CV values of 70 and 85%.

Discussion

Salient outcomes of the present investigation are that, first, in young men GH secretory-burst shape (waveform) can be modeled as a flexible three-parameter probability distribution that allows for variably asymmetric waveforms. Secondly, compared with saline, dual secretagogues induce more rapid initial GH release within secretory bursts as quantified by a median 43% abbreviation of the time latency to maximal secretion. Thirdly, the GH pulse-renewal process in men is associated with a mean interburst interval of about 50 min, wherein the pulsing variability (CV=75%) is less than that of a classical Poisson process (CV=100%). Fourthly, basal (non-pulsatile) GH secretion in healthy men not infused with any secretagogue accounts for ~12% of total fasting GH secretion. And fifthly, short-term depletion of gonadal sex steroids achieved by leuprolide administration does not modify GH secretory-burst shape, the efficacy of peptidyl secretagogues, or the frequency and variability of GH pulses. By contrast, short-term hypogonadism reduced (non-pulsatile) GH secretion by 60% compared with eugonadism. The collective data indicate that GH secretory-burst waveform is determined by secretagogue input, but not sex-hormone milieu; basal GH secretion depends upon gonadal-steroid availability; and the GH-pulsing mechanism is stable to short-term sex-steroid deprivation.

The principal effects of paired secretagogues in healthy men were to augment the amount (mass) of GH secreted in bursts by 16- to 44-fold and accelerate initial burst-like GH release by 1.8-fold. Both effects were independent of profound sex-steroid depletion achieved with a synthetic GnRH agonist. In the absence of secretagogue infusions, secretory bursts were characterized by a rapid initial increase in GH release, which was maximal after 20 min. Secretion rates then declined slowly, and continued to exceed 5% of the maximum for an additional 65-90 min. Albeit quite marked, asymmetry of GH secretory bursts was not recognized in earlier studies in men, given that secretory episodes were assumed *a priori* to have a symmetric Gaussian shape (25-27).

Atomic force microscopy has demonstrated that rapid release of GH involves fusion of docked exocytotic vesicles with cell-membrane pores, followed by partial or complete discharge of granule contents into extracellular fluid (28). Continuing peptide secretion requires more prolonged and/or more frequent fusion of exocytotic vesicles with the plasma membrane as well as synthesis, delivery, docking, and fusion of newly packaged granules (3-6). Based upon these concepts, we postulate that GHRH and GHRP accelerate initial GH release by enhancing early steps in exocytosis, and concomitantly prolong GH secretion after the burst maximum by enhancing the production and recruitment of new secretory granules. If this assessment is valid, then previous methodologies that did not allow for asymmetric secretory bursts would have systematically underestimated pulsatile and overestimated basal secretion for any given hormone half-life.

The pulse-renewal (pulse-generating) process that underlies the random sequence of interburst intervals was approximated by a Weibull probability density. The two-parameter Weibull distribution allows greater flexibility in representing pulsing mechanisms than a one-parameter Poisson model (9). Flexibility is achieved by the parameters λ (mean pulsing frequency) and γ (variability of the pulsing intervals). In particular, $\gamma>1.0$ in the Weibull process allows for lesser variability in pulse timing than 100%, as required definitionally in the Poisson distribution where γ equals 1.0. For the GH time series studied here in men, γ values were 1.2 and 1.4, which correspond to interpulse-interval variability of 70-85%. Similar variability was estimated for the GH-pulsing mechanism in young women (14), but markedly less variability was inferred for GH in postmenopausal women (γ 2.2-2.4) and LH in men (2.5-3.7) and women (2.4-5.4) (12,29,30). The Weibull model of pulsatility further revealed that short-term sex-steroid deprivation does not detectably disrupt the GH pulse-renewal process.

Basal (non-pulsatile) GH secretion was 2.5-fold higher in EU than HYPO men. The mechanisms mediating this difference are not known. Randomization bias appears unlikely to explain this. A plausible postulate is that sex-steroid depletion increases either basal (non-pulsatile) hypothalamic somatostatin (SS) secretion or pituitary SS-receptor (SSTR) expression. The latter consideration would be consistent with the fact that E_2 can repress expression of the pituitary *SSTR5* gene in the rodent, and augment inhibition of GH secretion by infused SS in the humans (31,32).

Qualifications include the need to corroborate outcomes in an independent cohort of subjects extend the duration of sampling to improve the precision of some parameter estimates, evaluate nonclassical modulators of GH secretion such as neuropeptide Y-Y2 receptor (NPY-Y2) (33), and verify analytical inferences directly by invasive sampling in a suitable animal model.

In summary, a variable-waveform deconvolution model was applied to GH-concentration time series in 22 young men who underwent stimulation with saline and 3 distinct secretagogue pairs. Analyses revealed time-asymmetric GH secretory bursts, which peaked in 20 min and continued for a total of 65-90 min in the unstimulated state. Peptidyl secretagogues reduced the time latency to maximal GH release by 43% and extended the duration of the secretory burst by an additional 20-30 min. Suppression of gonadal sex steroids for 31-39 days reduced basal (non-pulsatile) GH secretion by 62%, but did not disrupt the GH secretory-burst waveform, alter the pulse-renewal process, or decrease the stimulatory effects of peptidyl secretagogues. We conclude that peptidyl secretagogues and gonadal sex steroids control highly distinct facets of GH secretion in men.

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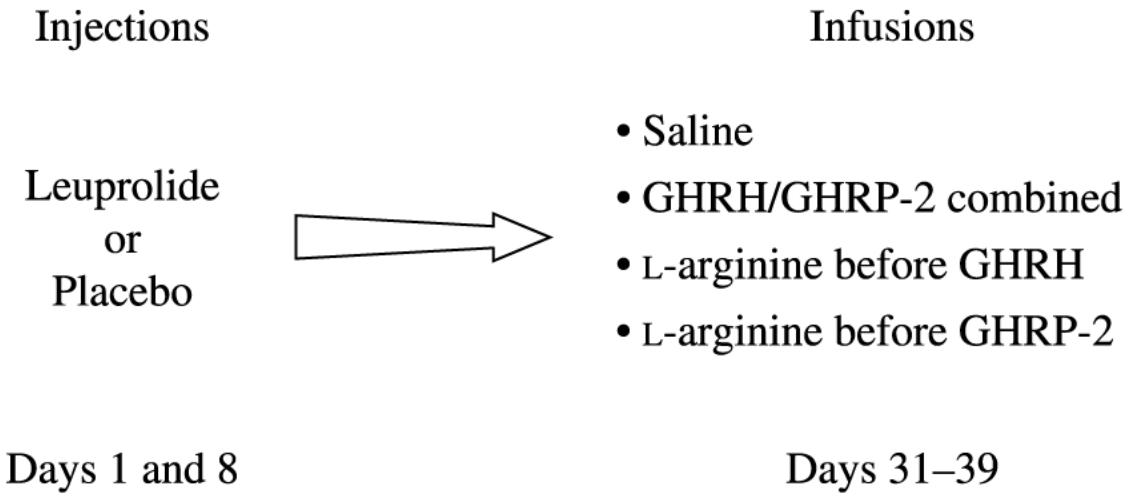

Figure 1.
Schema of study protocol designed to examine the impact of secretagogues and sex-steroid milieus on the GH secretory process in men.

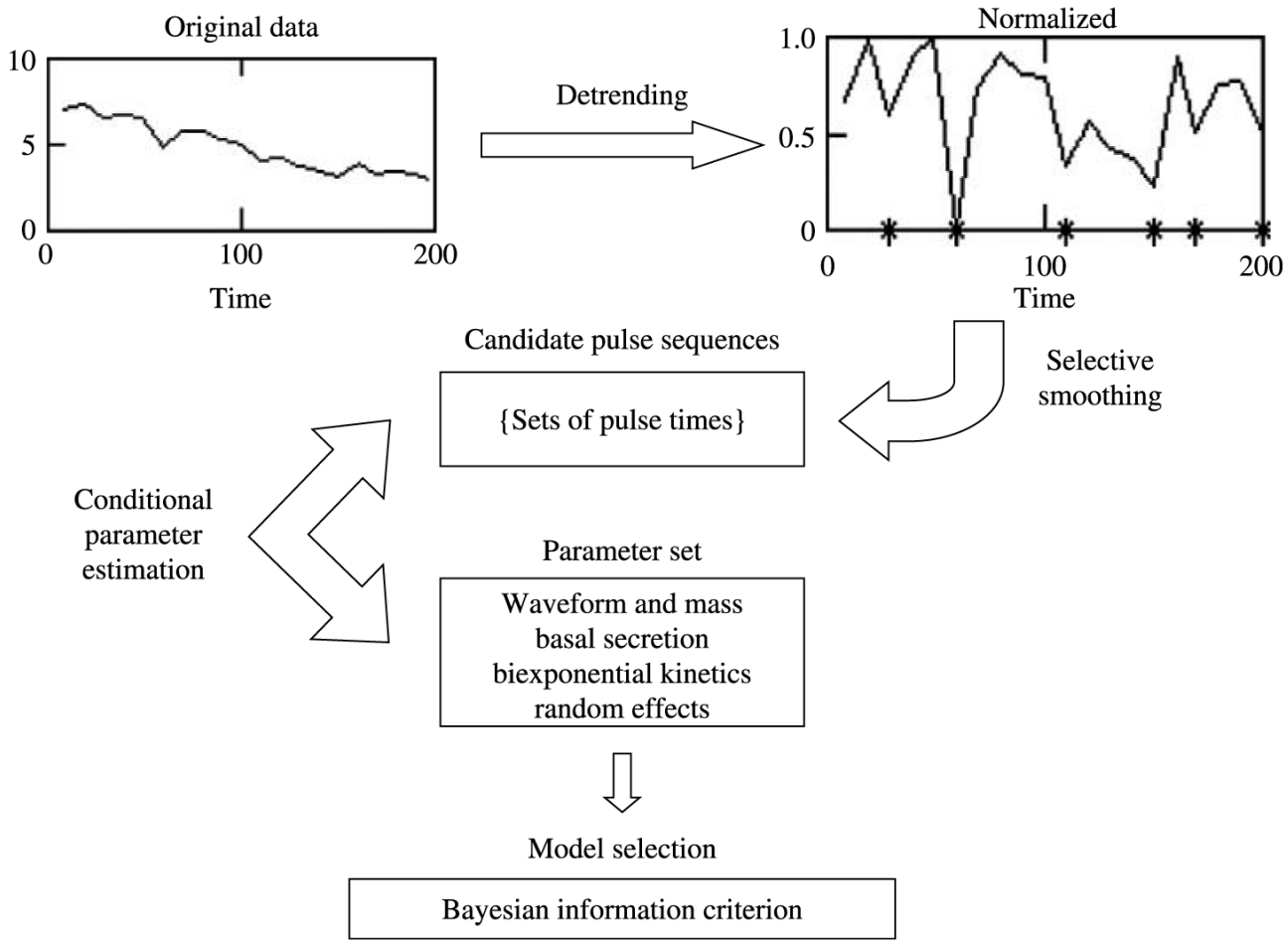

Figure 2.

Analytical model of variable-waveform deconvolution analysis. The methodology requires: (i) detrending and normalizing the hormone concentration time series to the unit uniform interval (0, 1) (top row) using the heat equation, which removes low-frequency terms without *a priori* specification of the number of terms; (ii) constructing decremental sets of potential GH pulse-onset times by selective smoothing (middle) using a nonlinear diffusion algorithm; (iii) estimating all secretion and elimination parameters simultaneously conditional on a given set of candidate pulse times (bottom); and (iv) model selection across candidate pulse-time sets based upon the Bayesian information criterion.

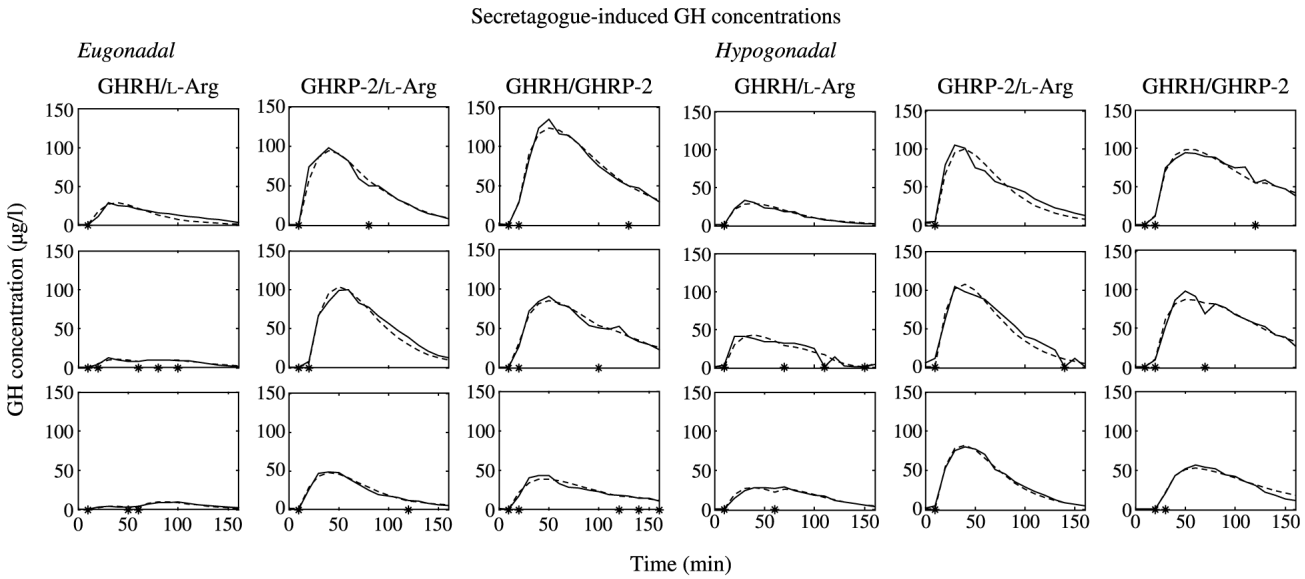

Figure 3.

Illustrative GH-concentration time series in three EU (eugonadal) and three HYPO (leuprolide-suppressed hypogonadal) men. Data are 10 min measurements for 1 h before i.v. infusion of (a) GHRH and GHRP-2 together each at a rate of 1 μ/kg per h for 2.5 h; (b) L-arginine (30 g over 30 min) followed by bolus GHRP-2 (3 μ/kg); and (c) L-arginine followed by bolus GHRH (1 μ/kg). Continuous curves denote measured GH concentrations, and interrupted curves deconvolution-estimated concentrations. Not shown are 6 h baseline (saline-infusion) sampling sessions in the 12 EU volunteers.

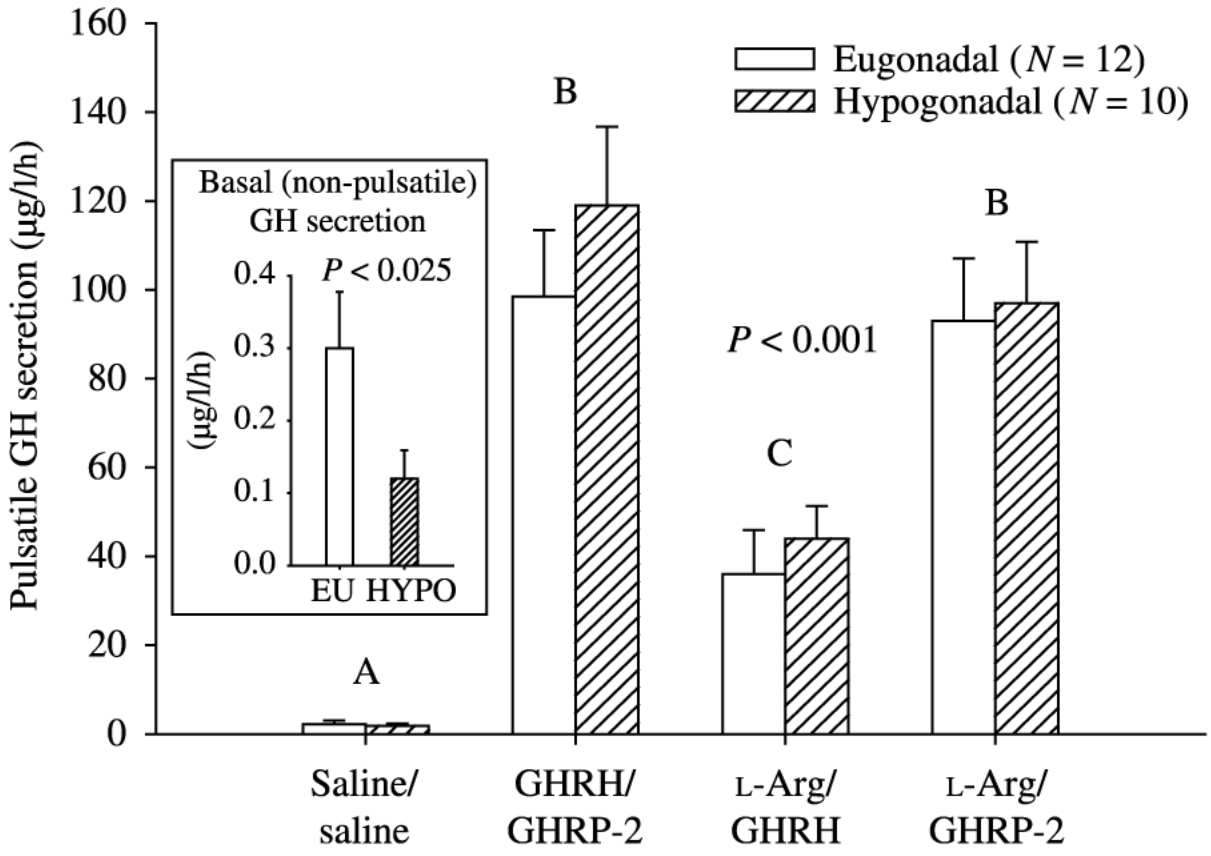

Figure 4.

Pulsatile GH secretion during i.v. infusion of saline, GHRH/GHRP-2, L-arginine/GHRP-2, and L-arginine/GHRH in 12 EU and 10 HYPO men. HYPO men received leuprolide 31-39 days earlier to deplete gonadal sex steroids. Data are the mean±S.E.M.. *P* defines the overall secretagogue effect. Different alphabetic superscripts designate significantly different GH responses assessed via Tukey's honestly significant difference multicomparison test. The inset graph gives basal (non-pulsatile) GH secretion, which differed in EU and HYPO at $P<0.025$.

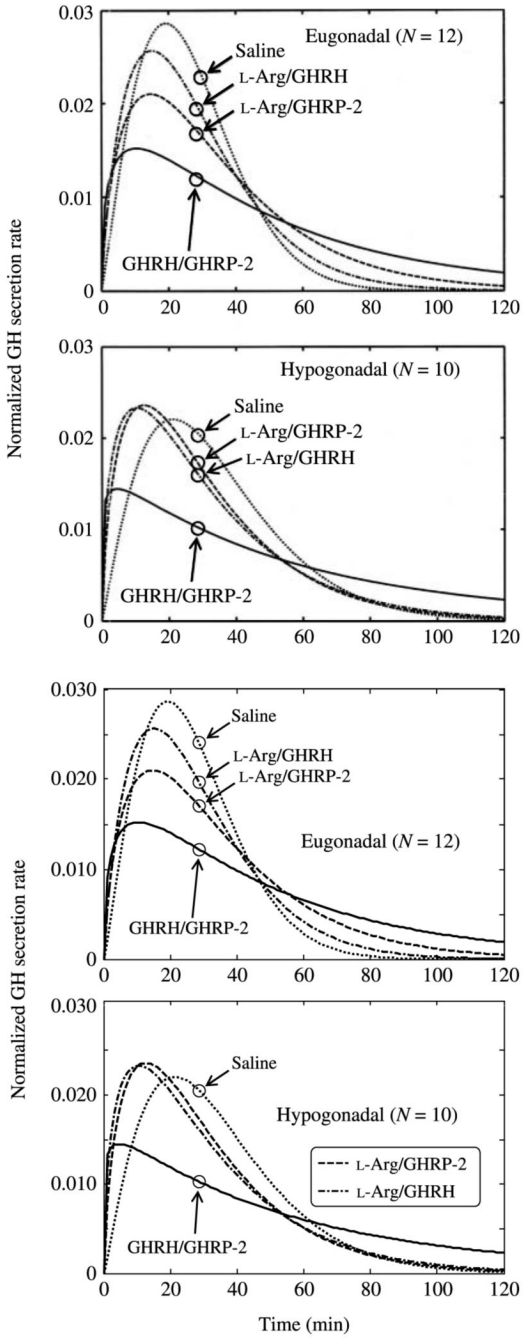

Figure 5.
Waveform (time evolution of instantaneous secretion rates) of GH secretory bursts in the four interventions (saline versus dual secretagogues) in cohorts comprising 12 EU (top) and 10 HYPO (bottom) men.

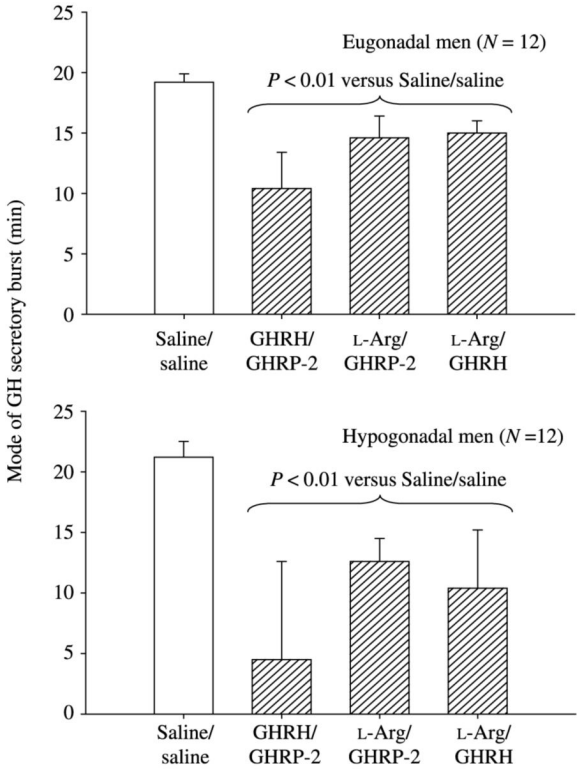

Figure 6.

Modes of GH secretory-burst waveform (approximated by a three-parameter generalized gamma probability distribution) defined as the time delay (min) from objective secretory-burst onset to maximal secretion. Data are the mean±S.E.M. presented as described in Fig. 4.

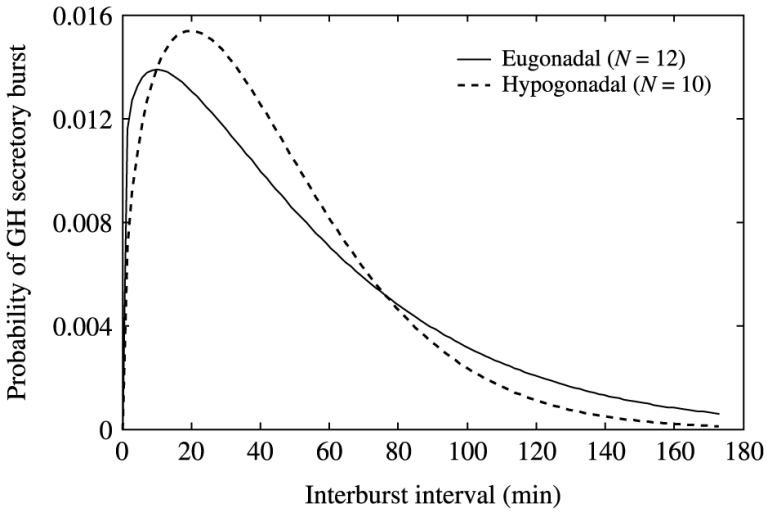

Figure 7.
Distribution of GH interpulse intervals (min) in 12 EU and 10 HYPO men estimated as a two-parameter Weibull-renewal process. Mean interpulse intervals and pulsing regularity did not differ in the two cohorts (Results).

Table 1

Hormone concentrations during sampling

Hormone	**Eugonadal (*N*=12)**	**Hypogonadal (*N*=10)**	***P* value**
LH (IU/l)	2.9±0.27	1.3±0.33	0.002
Testosterone (nmol/l)	18.8±1.7	1.8±0.042	<0.001
Estradiol (pmol/l)	92±7.7	31±3.0	<0.001
IGF1 (μg/l)	384±22	425±56	>0.10

Data are the mean±S.E.M. *P* values were estimated via an unpaired one-tailed Student's *t*-test.

ERRATUM

Secretagogues govern GH secretory-burst waveform and mass in healthy eugonadal and short-term hypogonadal men

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The authors and the journal apologize for an error in the above paper which appeared in **159** (5) 547–554. On page 553 the correct sentence should read 'The latter consideration would be consistent with the fact that E2 can repress expression of the pituitary SSTR5 gene in the rodent, and relieve inhibition of GH secretion by infused SS in the humans (31, 32)'.

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