Regulated recovery of pulsatile growth-hormone secretion from negative feedback: a preclinical investigation

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Abstract

Although stimulatory (feedforward) and inhibitory (feedback) dynamics jointly control neurohormone secretion, the factors that supervise feedback restraint are poorly understood. To parse the regulation of growth-hormone (GH) escape from negative feedback, 25 healthy men and women were studied 8 times each during an experimental GH-feedback clamp. The clamp comprised combined bolus infusion of GH or saline and continuous stimulation by saline GHRH, GHRP-2 or both peptides after randomly ordered supplementation with placebo (Pl, both sexes) vs E₂ (women) and T (men). Endpoints were GH pulsatility and entropy (a model-free measure of feedback quenching). Gender determined recovery of pulsatile GH secretion from negative feedback in all four secretagogue regimens (0.003 ≤ P ≤ 0.017 for women > men). Peptidyl secretagogue controlled the mass, number and duration of feedback-inhibited GH secretory bursts (each P < 0.001). E₂/T administration potentiated both pulsatile (P = 0.006) and entropic (P < 0.001) modes of GH recovery. IGF-I positively predicted the escape of GH secretory-burst number and mode (P = 0.022), whereas BMI negatively forecast GH secretory-burst number and mass (P = 0.005). The composite of gender, BMI, E₂, IGF-I and peptidyl secretagogue strongly regulates the escape of pulsatile and entropic GH secretion from autonegative feedback. The ensemble factors identified in this preclinical investigation enlarge the dynamic model of GH control in humans. **Word Count: 201**
Introduction

Endocrine glands signal remote tissues via a predominantly pulsatile mode of hormone secretion (54). Regulated pulsatility is obligatory for gonadotropin-releasing hormone’s drive of gonadotropin pulses (28; 38). For the experimentalist, the frequency and amplitude of hormone pulses provides a window into intermittent hypothalamic inputs to the anterior pituitary gland (11; 35). Moreover, in the case of GH secretion, pulsatility confers a powerful mechanism for directing gender-selective gene expression in target tissues, such as liver, muscle, fat and brain (13; 16; 22; 56; 57).

Negative feedback is a critical component of dynamic control in diverse endocrine systems (5; 52), preventing excessive hypothalamic and/or pituitary secretion as systemic hormone concentrations rise. Simplified mathematical models support the intuition that negative feedback with a time delay would be sufficient to both sustain and modulate GH pulsatility (9). Therefore, relative hyposomatotropism in aging, obesity or gonadal insufficiency and relative hypersomatotropism in puberty could in principle reflect reciprocal adaptations in GH feedforward (stimulation) and GH feedback (restraint). Despite the importance of negative feedback in hormonal dynamics (13; 18; 34; 54), the precise mechanisms that direct GH’s recovery from autoinhibition in the human have been difficult to elucidate. The present analyses test the thesis that factors presumed to modulate GH feedforward, viz. gender, BMI, estradiol (E2), insulin-like growth factor (IGF-I) and peptidyl secretagogues, jointly regulate the recovery of pulsatile GH secretion from negative feedback in healthy individuals.

Methods

Subjects
Twenty-five healthy older adults participated (N=14 men, N=11 women) after providing witnessed voluntary informed consent approved by Mayo Institutional Review Board. Data from two subsets of subjects were evaluated earlier in relation to the recovery of algebraic nadir and peak GH concentrations and of approximate entropy (ApEn) during saline and 2-peptide infusion (45; 46). Deconvolution analyses were performed here for the first time. The safety of the protocol was reviewed by the US Food and Drug Administration under an investigator-initiated new drug application for infusion of the experimental peptides, GHRH-1,44-amide (GHRH) and the GH-releasing peptide, GHRP-2 (2). Exclusion criteria were any systemic, metabolic, inflammatory or organ-level disease; administration of neuropsychiatric drugs; hormone exposure (except L-thyroxine with normal TSH during study); recent weight loss; major stress (job loss, divorce, death in family); alcohol abuse or illicit drug use; and failure to provide informed consent. Inclusion criteria were age ≥45 yr, healthy, community-dwelling, ambulatory, consenting adults with 18<BMI <35 kg/m². Older adults were evaluated, given that the stimulatory effect of testosterone (T) is more prominent in elderly than young men (12), and that of estradiol (E₂) is highly consistent in postmenopausal women (40). All women were biochemically postmenopausal (E₂ < 15 pg/mL, FSH > 50 IU/L, LH > 25 IU/L).

Protocol

The study was a double-blind, randomized cross-over design. Each subject was studied 8 times fasting, four times while receiving placebo and four times while receiving E₂ (women) or T (men) supplementation with a 4-wk washout before and between sex-steroid exposure. Each study session comprised 6-min bolus i.v. injection of GH (1
µg/kg) at 0830 hr to impose negative feedback and concurrent blood sampling every 10 min beginning one-half hr before the bolus (i.e., 0800 hr) for a total of 8 hr (until 1600 hr). Sessions were conducted at least 48 hr apart. Feedback escape was monitored during separate continuous constant i.v. infusion of saline (20 mL/h), GHRH (1 µg/kg/h), GHRP-2 (1 µg/kg/h) and combined GHRH/GHRP-2 (both 1 µg/kg/h) from 0800 to 1600 hr. Infusion sessions were scheduled during the time window inclusive of days 11 to 21 (day 1 was start of placebo/E2/T). Sex steroid supplementation included estradiol-17 beta 1 mg orally twice daily for 21 days in women, and 200 mg testosterone enanthate i.m. every 10 days in men, since these treatment schedules have been shown to stimulate fasting GH secretion without imposed negative feedback (12; 40). Placebo for E2 was an identical capsule without E2, and placebo for T was 1 mL saline injection.

Assays

GH was measured by robotics-automated chemiluminescence double-antibody assay (Beckman Access Dxl, Beckman Coulter, Fullerton, CA) and total IGF-I by immunoradiometric assay (Diagnostic Systems Laboratory, Webster, TX), exactly as described (15). E2 and T were quantified by LC-MS/MS, as reported earlier by the Mayo mass spectrometry laboratory (27; 42). Respective sensitivities were 0.010 µg/L (GH), 2.8 pg/mL (E2) and 5 ng/dL (T). IGFBP-I (a secondary marker of E2 action) and IGFBP-3 (a marker of GH action) were assayed as possible covariates of regulated GH secretion, as described (50).

Deconvolution analysis

The primary outcome of deconvolution analysis was pulsatile GH secretion during feedback recovery (1030-1600 hr). This 5.5-hr time interval was chosen to allow at least
5 half-lives of exogenous GH decay after i.v. injection at 0830 [6.7 half-lives expire over the 120 min between 0830 and 1030 hr assuming a nominal GH half-life of 18 min (54)]. Pulsatile secretion is the sum of GH secretory-burst mass (µg/L) values (52). Secondary outcomes of basal (nonpulsatile) GH secretion, GH secretory-burst frequency (number per 5.5 hr) and shape (modal time delay from pulse onset to maximum GH secretion within bursts) were estimated concomitantly. With respect to pulse detection, sensitivity and specificity are both 0.93 (23).

Approximate entropy (ApEn)

Approximate entropy, ApEn (1, 20%), was used as a scale- and model-independent regularity statistic to quantify the orderliness (regularity) of hormone release (14; 31). Higher ApEn denotes greater disorderliness (irregularity) of the secretion process. Mathematical models and clinical experiments establish that greater irregularity signifies decreased feedback control with high sensitivity and specificity (both > 90%) (33; 55).

Statistics

The principal hypothesis posed a priori was that recovery of pulsatile GH secretion during experimental negative feedback is jointly determined by gender, sex-steroid treatment and peptide secretagogue. This postulate was tested in two ways. First, 3-way ANCOVA was employed to distinguish the categorical effects of E₂/T vs placebo treatment (2 factors), gender (2 factors) and peptidyl secretagogue type (saline + 3 peptidyl factors). Pulsatile GH secretion on the placebo/saline control day was employed as the covariate. To stabilize residual variance, dependent variables were subjected to natural-logarithmic transformation. The variance-covariance matrix was modeled in a compound symmetry form. Parameters were estimated by residual
maximum likelihood. Two and three-way interactions were modeled concurrently. Model
P and $R^2$ were estimated at each step, and Tukey’s honestly significantly different
(HSD) test was applied post hoc at protected experiment-wise two-tailed $P<0.05$ (10).
Second, for each infusion type, exploratory (hypothesis-generating) stepwise
multivariate backward-elimination regression analysis was employed with an expanded
independent-variable set of gender, BMI, $E_2$, T, IGF-I, IGFBP-1 and IGFBP-3
concentrations, using SYSTAT 11 (SYSTAT Software, Richmond, CA, 94804). Alpha to
remove a variable was 0.05. Standardized (slope) coefficients were calculated as linear-
regression coefficient/standard deviation of the independent variable. Data are
expressed as the geometric mean ± SEM. The geometric mean is the antilogarithm of
the algebraic mean of the individual logarithms.

Results

When women received placebo, $E_2$ averaged $9.9 \pm 0.36$ pg/mL (highest value 12
pg/mL) and when they received $E_2$ supplementation $E_2$ averaged $114 \pm 18$ pg/mL
($P<0.001$ treatment effect). Progesterone was < 1 ng/dL in all. In men, placebo and T-
supplementation yielded total T concentrations of $384 \pm 29$ and $859 \pm 68$ ng/dL
($P<0.001$). $E_2$ in men rose from $27 \pm 1.8$ to $83 \pm 7.6$ pg/mL ($P<0.01$). The median
(range) of age in women was 58 (50-74) yr and in men 53 (45-74) yr ($P=0.26$).

Primary outcomes of deconvolution analysis were pulsatile (summed mass of GH
secreted in bursts), basal (nonpulsatile), and total (pulsatile plus basal) GH secretion.
According to ANCOVA, all 3 of peptide ($P<0.001$), gender ($P<0.001$) and $E_2$/T treatment
($P=0.006$) determined pulsatile GH secretion during feedback recovery [overall-model
and covariate P values both <0.001 with multivariate $R^2=0.73$]: Figure 1. Specifically,
the descending rank order of mean peptide effects was combined peptides
>>GHRH=GHRP-2>>saline for the 25 subjects studied (top panel). The main gender
effects were due to greater pulsatile GH secretion in women than men under dual-
peptide stimulation (P=0.001) [upper middle] and independently of infusion type in the
placebo setting (P=0.012) [lower middle]. The treatment effect was attributed to E₂'s
stimulation of pulsatile GH in women over each of placebo in men (P<0.001), T in men
(P<0.001) and placebo in women (P=0.051). There was no gender x peptide (P=0.377)
or gender x treatment (P=0.308) interaction (upper middle, lower middle). Treatment x
peptide interacted at P<0.001 (bottom), reflecting higher pulsatile GH secretion during
saline (but not peptide) infusion in the presence of E₂/T compared with placebo
(P<0.001). Tukey's HSD test revealed greater pulsatile GH recovery in women than
men for both placebo and E₂/T-supplemented GHRH/GHRP-2 infusions
(0.004≤P≤0.034).

Basal (nonpulsatile) GH secretion was also assessed by ANCOVA (P<0.001 overall,
R²=0.82, P<0.001 covariate effect). Unlike pulsatile GH recovery, basal GH recovery
was determined by peptide (P<0.001) and E₂/T treatment (P=0.022), but not by gender
(P=0.855): Figure 2. Basal GH secretion decreased in the rank order of combined
peptides>GHRP-2>GHRH>saline infusion whether placebo or E₂/T was administered
(top). The effect of GHRP-2 stimulation exceeded that of GHRH in men but not women
(upper middle), yielding a gender x peptide interaction (P<0.001). There was a weak
gender x treatment effect (P=0.053) due to stimulation by E₂ in women but not by T in
men (lower middle). Sex-steroid treatment compared with placebo elevated overall
basal GH secretion (P=0.022), but not significantly for any single-infusion type. Post hoc
gender comparisons disclosed greater basal GH secretion in women than men during placebo/GHRH ($P \leq 0.022$) and placebo/GHRP-2 ($P=0.016$).

Total (basal plus pulsatile) GH secretion is a measure of overall GH escape under controlled negative feedback. By 3-way ANCOVA, model and covariate $P$ values were both $<0.001$ with $R^2=0.85$ due to strong main effects of peptide ($P<0.001$), gender ($P<0.001$) and treatment ($P<0.001$): Appendix Figure 1. The rank order of total GH secretory recovery was GHRH/GHRP-2>>GHRP-2=GHRH>>saline. The main gender difference was women>men for GHRH infusion ($P=0.005$). Weak gender differences involved greater total GH recovery in women than men for placebo/GHRH ($P \leq 0.043$) and $E_2/T/GHRH/GHRP-2$ ($P \leq 0.034$). Sex-steroid treatment yielded higher overall GH recovery than placebo during saline infusion ($P<0.001$). There was no gender x treatment interaction ($P=0.124$), but there were interactions for gender x peptide ($P=0.017$) and treatment x peptide ($P<0.001$). By post hoc Tukey’s test, these interactions reflected (i) the absence of a $T$ effect in men despite an $E_2$ effect in women over placebo ($P=0.001$), and (ii) an $E_2/T$ effect over placebo during saline but not peptide infusion ($P<0.001$).

To elucidate the mechanisms of enhanced pulsatile GH recovery, analyses were extended to include GH secretory-burst mass (size), number (frequency) and shape (mode). GH secretory-burst mass was determined jointly by gender ($P<0.001$) and peptide type ($P<0.001$), but not $E_2/T$ treatment ($P=0.159$) [overall ANCOVA model $P<0.001$, $R^2=0.69$, covariate $P<0.001$]: Table 1 (column 2) and Appendix Table 1. Compared with placebo/saline, GHRH and GHRP-2 infusions augmented recovery of burst mass by 9.1-9.7-fold (placebo) and by 8.1-8.3-fold ($E_2/T$), with no difference
between the peptides. The effects of combined GHRH/GHRP-2 stimulation were 20-fold (placebo) and 22-fold (E2/T) that of saline, thus exceeding the individual effects of GHRH and GHRP-2 (Table 1). Gender effects on burst mass were due to: (i) larger bursts during GHRH and combined GHRH/GHRP-2 infusion in women than in similarly infused men independently of sex-steroid milieu (P=0.030 gender x peptide interaction); (ii) higher burst mass in women than men given placebo (P=0.017); and (iii) bigger bursts in women given E2 than in men given either T (P=0.002) or placebo (P<0.001). A marked treatment x peptide interaction (P=0.001) was explained by greater GH secretory-burst mast in the E2/T than placebo condition during saline infusion (P=0.001). The highest secretory-burst mass was attained in women receiving combined GHRH/GHRP-2 independently of sex-steroid treatment (P<0.001). Post hoc gender comparisons revealed higher GH secretory-burst mass in women than men under placebo/GHRH, placebo/GHRH/GHRP-2, and E2/T/GHRH/GHRP-2 (0.005≤P≤0.04).

Three-way ANCOVA of GH secretory-burst number during GH autofeedback revealed overall model P<0.001, R²=0.27, and covariate (P<0.001) significance due to main effects of peptide type (P<0.001) and E2/T treatment (P=0.019), but not gender (P=0.403): Table 1 (column 3). There were no pairwise interactions (P>0.10):

Appendix Table 1. By Tukey’s test, the descending rank order of recovery of GH secretory-burst frequency was GHRH=combined-peptide infusion>GHRP-2>saline. In particular, burst number for GHRH exceeded that for GHRP-2 (P=0.032). Sex-steroid effects were attributable to higher frequency in E2/T/GHRH than in each of placebo/saline (P<0.001), E2/T saline (P=0.005), or placebo/GHRP-2 (P=0.003). Although gender had no effect on this measure, only men manifested a higher GH burst
frequency during GHRH (P<0.001) and GHRH/GHRP-2 (P=0.001) compared with saline infusion.

GH secretory-burst mode was used as a measure of burst shape, with smaller values (min) corresponding to more rapid onset of maximal GH release. The ANCOVA model (overall P<0.001, R²=0.23, covariate P=0.086) showed a main effect of peptide type only (P<0.001): Table 1 (column 4) and Appendix Table 1. Specifically, combined GHRH/GHRP-2 stimulation abbreviated GH secretory bursts in the placebo setting (mode 12±0.68 min) compared with (a) both placebo/saline and E₂/T saline (both P<0.001 for modes of 16±1.2 and 15±0.8 min) and (b) placebo/GHRP-2 (P=0.002 vs mode 16±0.61 min). Independently of placebo vs E₂/T treatment, dual-peptide stimulation evoked shorter GH-secretory bursts than GHRP-2 (P=0.012). There were no gender effects by Tukey’s test.

Exploratory stepwise backward-elimination multivariate regression was applied to test the hypothesized impact of gender, BMI, and E₂, T, IGF-I and IGFBP concentrations on feedback escape. Table 2 summarizes key outcomes for pulsatile GH secretion. Under feedback during placebo/saline administration, prominent joint determinants of pulsatile GH secretion were gender (women>men, P=0.003), E₂ (positive, P=0.018), T (positive, P=0.007), IGF-I (positive, P=0.001) and BMI (negative, P=0.013) (P overall<0.001). The 5 factors together explained nearly 70% (R²=0.684) of the variance in the group of 25 subjects. To visualize several key interactions, Figure 3 depicts three 3-dimensional surfaces relating pulsatile GH escape (dependent variable) to independent-variable pairs: viz., E₂ x BMI, E₂ x IGF-I and IGF-I x BMI (top, middle and bottom panels).

Comparisons of standardized regression coefficients revealed that each SD increase in
E₂, IGF-I and BMI was associated with a 0.748 increase, a 0.590 increase and a 0.430 decrease in pulsatile GH secretion (µg/L/5.5 hr), respectively. These values compare with standardized regression coefficients of 1.327 for women>men and 1.050 for T effects. There were no significant multivariate associations under placebo/GHRH or placebo/GHRP-2 (all P>0.05). For placebo/GHRH/GHRP-2, the independent variables IGFBP-1 and IGFBP-3 together accounted for 40% of the variance in pulsatile GH recovery (P=0.004, R²=0.400) with positive effects in both cases (Table 2).

Prominent gender differences in pulsatile-GH recovery emerged in the E₂/T/saline and E₂/T/GHRH/GHRP-2 settings by multivariate regression, in which women secreted significantly more GH in pulses than men (both P=0.003, R²=0.406 and 0.318 respectively). During saline infusion, IGF-I and IGFBP-1 were also positive determinants, whereas during combined-peptide infusion, only gender (women>men) controlled pulsatile GH recovery (Table 2). In addition, pulsatile GH output was related to E₂ concentrations positively under E₂/T/GHRH (P=0.026) and E₂/T/GHRP-2 (P=0.037).

Correlates of total GH recovery followed the pattern of pulsatile GH secretion, wherein total GH secretion was related to: (i) all 3 of E₂ (P=0.005, beta=0.128), IGF-I (P=0.044, beta=0.012) and BMI (P=0.004, beta=-0.459) for placebo/saline [overall P=0.008, R²=0.474]; (ii) gender (P=0.033, women>men) for placebo/GHRH; (iii) E₂ (P=0.001, beta=-0.486) for placebo/GHRH/GHRP-2; and (iv) E₂ (P=0.007, beta=0.219) for E₂/T/GHRP-2.

Viewed with respect to mechanistic measures of GH secretion, multivariate regression on the placebo/saline day showed: (1) greater GH secretory-burst mass with increased
E₂ concentrations and decreased BMI: Appendix Table 2 [column 2] and Figure 4 (top); and (2) increased GH burst number with higher IGF-I and lower BMI [column 3]: Figure 4 (bottom). Women had more prolonged GH secretory bursts (increased mode) than men in the E₂/T setting during saline, GHRH, and combined GHRH/GHRP-2 infusions, and the opposite during GHRP-2 infusion under E₂/T treatment [Appendix Table 2 column 4] and Figure 5 (top). Basal (nonpulsatile) GH secretion was positively determined by T and IGFBP-1 together (P<0.001, R²=0.484) under saline/placebo (P=0.007) and during GHRP-2 infusion/placebo (P=0.003): Figure 5 (bottom). IGFBP-1 was also a direct correlate of basal GH output during saline (P<0.001) and GHRH (P=0.009) infusion/placebo. During E₂/T/GHRH, E₂ positively forecast basal GH escape (P=0.018).

Approximate entropy (ApEn), a measure of serial irregularity and an inverse metric of feedback strength, was greatest in the GHRP-2 setting under E₂/T replacement, exceeding that under all other conditions except combined GHRH/GHRP-2 drive whether after placebo or E₂/T: Figure 6. ApEn was positively associated with E₂ (P=0.002) and female sex (P=0.011) in the placebo/GHRH/GHRP-2 context. In the placebo/GHRP-2 setting, IGFBP-1 was a negative correlate of GH ApEn (R²=0.280, P=0.007): Appendix Table 2 (column six).

Discussion

Negative feedback and positive feedforward represent codominant mechanisms for regulating pulsatile hormone secretion in the reproductive, somatotropic and stress-adaptive axes (17; 18; 34; 54). Virtually nothing is known about the physiological and preclinical factors that supervise time-varying feedback strength. As a general paradigm
for feedback control, the present work exploits specific and precise measures of pulsatile and entropic GH secretion during escape from autoinhibition in healthy adults. A salient thematic outcome of deconvolution and ApEn analysis was that multiple factors interact in determining quantifiable escape from exogenously controlled feedback. Most prominent are strong effects of gender, relative adiposity (BMI), E₂, T and IGF-I concentrations on GH secretory recovery in the placebo/saline paradigm. Specifically, recovery of pulsatile GH secretion under endogenous peptide drive was determined jointly by gender (women > men), E₂ (std coeff 0.748), T (std coeff 1.05), IGF-I (std coeff 0.59), and BMI (std coeff -0.43), together explaining 68% of intersubject variance. The positive effect of female gender and endogenous E₂ and T on feedback escape might be explained by the capability of estrogen to potentiate stimulation by submaximally effective amounts of GHRH and ghrelin (1; 19; 48; 51) and attenuate inhibition by exogenous IGF-I and somatostatin (3; 50). These mechanistic inferences depend upon the assumption that exogenous and endogenous GHRH and ghrelin exert similar effects. The stimulatory association with T could parsimoniously reflect T’s aromatization to E₂ (13). The positive relationship with total IGF-I concentrations is concordant with the capability of higher pulsatile GH drive to mediate greater IGF-I and IGFBP-3 production (54). In this construction, IGF-I concentrations constitute a surrogate marker of baseline hypothalamopituitary drive of pulsatile GH output. The novel negative association between BMI and feedback recovery could arise from putative inhibitory effects of adipocytokines, free fatty acids, insulin and/or free IGF-I on pulsatile GH secretion (13; 21; 54). Whatever the definitive mechanisms, a tetrapartite interaction among gender, sex steroids, IGF-I and BMI accounted for more than two-
thirds of the interindividual variability of pulsatile GH’s escape from negative feedback in the absence of exogenous secretagogues. Pulsatile GH is of major importance as an anabolic signal in various species (13; 54).

Whereas recovery of GH secretion from autofeedback during continuous saline infusion monitors opposing somatostatin outflow (feedback) and endogenous secretagogue drive (feedforward), recovery of GH secretion during constant combined GHRH/GHRP-2 infusion should primarily mirror the opposing effects of somatostatin outflow to the pituitary gland. This is because neither E₂ nor T potentiates maximal stimulation by GHRH, GHRP-2, GHRH/GHRP-2 or native ghrelin (4; 7; 39; 44; 47). Estimated GH secretory-burst mass in the placebo/GHRH/GHRP-2 setting (12±1.9 µg/L) exceeded that in the placebo/saline setting (0.59±0.24 µg/L) by 20-fold under negative feedback. Similar absolute effects occurred during E₂/T supplementation. In contrast, the maximal difference in detectable burst number was less than 2-fold (3.7 vs 2.2 bursts/5.5 h). Therefore, recovery of pulsatile GH from autofeedback is primarily determined by augmentation of secretory-burst mass (90%) rather than frequency (10%). The combined-peptide effect probably includes direct pituitary stimulation as well as reduced somatostatin action at the pituitary level, given that neither secretagogue alters hypothalamic somatostatin release in animals (54). However, whether GH feedback-evoked hypothalamic somatostatin outflow is curtailed by GHRP/ghrelin is not known.

In contrast to pulsatile GH secretion, feedback-inhibited basal (nonpulsatile) GH secretion was determined positively by both T and IGFBP-1 concentrations in the placebo/saline setting, together explaining 48% of the variability in basal GH secretion
(P=0.001). T was also a positive correlate of basal GH output in the placebo/GHRP-2 context, and IGFBP-1 in the placebo/GHRH context. Positive covariance between basal GH release and IGFBP-1 concentrations could signify that this binding protein attenuates IGF-I’s inhibition of GH secretion (49). A positive effect of E₂ during E₂/T/GHRH drive might be due to the capability of estrogen to elevate basal GH secretion even in the absence of infused secretagogues (50). In mechanistic terms, somatostatin-receptor subtype 1 can mediate in vitro inhibition of basal GH secretion (20), and E₂ represses expression of this receptor subtype (54). Thus, decreased somatostatin action is plausible mechanism for elevated basal GH secretion by E₂. Conversely, E₂ can induce the GHRP receptor 1a, which otherwise is strongly constitutively expressed (29; 30). These findings permit a clear distinction between factors regulating pulsatile (sex-steroids, gender, BMI, IGF-I) and basal (sex steroids and IGFBP-1) GH escape from feedback.

During E₂/T supplementation and GHRH/GHRP-2 infusion, gender (women>men) independently of T, E₂, IGF-I, IGFBP-1/3 and BMI strongly (R²=0.318, P=0.003) predicted feedback-restrained recovery of pulsatile GH secretion. The sex difference was due to larger GH-secretory bursts in women than men. This outcome was not evident during placebo supplementation, suggesting that low endogenous E₂/T levels mask the gender contrast. Dose-response studies of E₂ and T action in men and women would be required to test this explanation of a gender difference in double-peptide drive. In addition, during 2-peptide stimulation E₂ may reduce somatostatin release/action more than T in humans. An alternative hypothesis is that nonsteroidal
gonadal factors also modulate GH secretion (25; 26; 41; 54), such as sex-chromosomal or neonatal imprinting of hypothalamo-pituitary responsiveness.

Gender contrasts also emerged with respect to GH secretory-burst shape, quantified by the modal time latency to maximal GH release within a burst. Burst mode was greater in men than women (P=0.001) and increased by E_2 administration (P=0.016) in the E_2/T/GHRP-2 setting. Enhanced recruitment within the pituitary somatotrope network and estrogentic induction of the GHRP-receptor 1a (but not the GHRH receptor) are potential mechanisms for this sex difference (30; 37). The gender effect was reversed during saline, GHRH and combined-peptide stimulation indicating specificity of the GHRP-2 effect. In an earlier study, 5 alpha-reduced androgen correlated with prolonged GH secretory bursts in men (53). Accordingly, metabolic products of T could mediate the sex difference in GHRP-stimulated secretory-burst waveform. To the degree that secretory-burst mode is a measure of exocytotically releasable GH vesicles (6), these data introduce the notion that gender, sex steroids and peptide secretagogue type determine somatotrope exocytosis.

BMI was a negative predictor of GH secretory-burst mass during GHRH infusion in the placebo (P=0.022) and E_2/T-supplemented milieu (P=0.008), explaining about 25% of the variance in this measure. This is significant in that >85% of GH secretion normally is pulsatile (13; 54). The inhibitory effect of BMI was robust, since it persisted after controlling for gender, E_2, T and IGF-I concentrations. In addition, BMI (negatively) and IGF-I (positively) together predicted GH pulse number during feedback suppression and placebo/saline rescue. The precise factors that mediate these inverse relationships between GH pulsatility and adiposity in humans are not yet known. Potential
mechanisms include accentuation of putatively suppressive effects of free fatty acids, IGF-I, insulin and TNF-alpha and/or attenuation of putatively stimulatory effects of adiponectin, resistin, eucortisolemia and leptin on GH secretion (24; 36; 43; 54).

ApEn (approximate entropy) provides a model-free and scale-invariant measure of altered feedback control in interlinked systems like the GHRH/ghrelin/somatostatin/GH axis (14; 55). Quite unlike female>male GH ApEn values in the fasting rat and human (32), gender had no effect on GH ApEn during feedback recovery (P=0.932 by ANCOVA). E2/T and GHRP-2 evoked higher GH ApEn (both P<0.001), which denotes reduction in quantifiable GH autofeedback (52). This was due principally to a joint E2/GHRP-2 effect (P=0.0081, R2=0.455). Because somatostatin mediates negative feedback, a parsimonious hypothesis is that E2 availability reduces somatostatin outflow and/or potentiates the capability of GHRP to antagonize hypothalamic somatostatin action, thereby yielding more irregular GH secretion patterns. The latter postulate is concordant with experimental data showing that GHRP inhibits brain actions of somatostatin (8; 13; 54). IGF-I feedback is indicated by the negative association of IGF-I concentrations with GH ApEn in GHRP-2-stimulated men and women (P=0.009, std coeff -0.620). The capability of E2/T to attenuate IGF-I feedback may thus be relevant also (49; 50).

Caveats include the relatively small cohort of 25 subjects (albeit representing a total of one-hundred 8-h sampling sessions), requiring confirmation in larger groups. The age range evaluated here did not include young adults, in whom sex-steroid levels are higher basally. The precise dose-response characteristics of GH’s feedback and E2/T’s potentiating effects are not yet known. Thus, further studies will be needed to elucidate
the dose dependencies of GH, sex-steroid and peptide-secretagogue effects on feedback. Body-compositional analysis and adipocytokine measurements would also aid in linking feedback correlates with specific fat depots.

In conclusion, each of gender, BMI, E₂, IGF-I and peptidyl secretagogue type controls feedback escape of pulsatile GH secretion. Specific determinants modulate GH secretory-burst mass, number and shape, and GH ApEn. Inasmuch as feedback (inhibition) and feedforward (stimulation) reciprocally govern GH secretion, the present preclinical investigations suggest a new level of complexity of GH regulation.

**Perspective and Significance**

Endocrine systems function as integrated networks, wherein homeostasis is maintained by recurrent, time-lagged adjustments in feedforward drive (stimulation) and feedback inhibition (repression) (52). The vast majority of extant studies have investigated stimulation or inhibition mechanisms individually. However, physiological pathways do not operate in isolation. Accordingly, a paradigm shift is to examine (controlled) feedforward under (controlled) feedback, allowing both processes to operate simultaneously. This concept is exploited here in the GH axis, wherein autofeedback can be imposed with and without secretagogue rescue. In principle, the idea could be applied to other physiological homeostatic systems by judicious selection of feedback and feedforward effectors.

**Disclosure Statement:** The authors have nothing to disclose.
Acknowledgments

We thank Jill Smith for support of manuscript preparation; Ashley Bryant for data analysis and graphics; the Mayo Immunochemical Laboratory for assay assistance; and the Mayo research nursing staff for implementing the protocol. Supported in part via the Center for Translational Science Activities (CTSA) Grant Number 1 UL 1 RR024150 from the National Center for Research Resources (Rockville, MD), and AG019695, AG029362 and DK050456 (Metabolic Studies Core of the Minnesota Obesity Center) from the National Institutes of Health (Bethesda, MD). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Aging or the National Institutes of Health. Matlab versions of the deconvolution methodology are available from Veldhuis.johannes@mayo.edu.
Reference List


   Structure and dynamics of the fusion pores in live GH-secreting cells revealed 

   Continuous 24-hour intravenous infusion of recombinant human growth hormone 
   (GH)-releasing hormone-(1,44)-amide augments pulsatile, entropic, and daily 
   rhythmic GH secretion in postmenopausal women equally in the estrogen- 
   withdrawn and estrogen-supplemented states. *J Clin Endocrinol Metab* 86: 700- 

8. Fairhall KM, Mynett A and Robinson IC. Central effects of growth hormone- 
   releasing hexapeptide (GHRP-6) on growth hormone release are inhibited by 

   sex differences in growth-hormone regulation in humans. *Am J Physiol Regul 

    58-74.


55. **Veldhuis JD, Straume M, Iranmanesh A, Mulligan T, Jaffe CA, Barkan A, Johnson ML and Pincus SM.** Secretory process regularity monitors


Figure Legends

Figure 1. Joint regulation of pulsatile GH secretion during negative feedback in 25 adults (14 men, 11 women). Subjects received a single bolus of GH i.v. to enforce feedback, and constant i.v. infusion of saline, GHRH, GHRP-2 or both peptides (each 1 µg/kg/h) to test feedback escape. Pulsatile GH secretion was estimated by deconvolution analysis. Three-way ANCOVA was applied to evaluate main effects of peptide type (top row) [P<0.001], gender (P<0.001) and sex-steroid treatment (P=0.006), as well as gender x peptide (upper middle), gender x treatment (lower middle) and treatment x peptide (bottom row) interactions. Overall P was <0.001 and R^2=0.73 for the model with covariate P<0.001. Within each row, means with no shared alphabetic letters differ by post hoc Tukey’s multiple-comparison HSD test. Thus, A differs from B and C, but not from AB or AC. Data are the geometric mean ± SEM.

Figure 2. Dual control of basal (nonpulsatile) GH secretion during feedback recovery in 14 men and 11 women by peptide type (P<0.001) and sex-steroid treatment (P=0.022) [overall ANCOVA model and covariate P<0.001, R^2=0.82]. The descending rank order of secretagogue effects was combined GHRH/GHRP-2>GHRP-2>GHRH>saline (top). There were significant interaction between gender and peptide (P<0.001, upper middle) and a strong interactive trend between gender and treatment (P=0.053, lower middle). Basal GH secretion was similar in women and men independently of peptide type across both sex-hormone milieus (upper middle); higher in women given E2 compared with placebo independently of peptide type (lower middle); and comparable in subjects given E2/T and placebo independently of peptide type and gender (bottom).

Superscripts are described in Figure 1.
**Figure 3.** Stepwise backward-elimination multivariate regression analysis of tripartite determination of pulsatile GH secretion by E2, BMI and IGF-I (overall $R^2=0.495$, $P=0.006$) under placebo/saline stimulation during negative feedback. The three 3-dimensional plots depict the joint influences of BMI and E2 (*top*), BMI and IGF-I (*middle*) and IGF-I and E2 (*bottom*). Standardized regression coefficients (std coeff) are the ratio of the multivariate regression slope to the variable’s SD. E2 and IGF-I positively, and BMI negatively, determined pulsatile GH recovery. Gender is indicated by open (male) and closed (women) circles.

**Figure 4.** Joint modulation of GH secretory-burst mass by E2 (positively, $P=0.006$) and BMI (negatively, $P=0.022$) in 25 adults during negative feedback in the placebo/saline setting [overall model $P=0.011$, $R^2=0.34$] (*top*). Two-variable control of GH secretory-burst number by BMI (negatively, $P=0.039$) and IGF-I (positively, $P=0.0043$) [overall model $P=0.013$, $R^2=0.37$] (*bottom*). Data are presented as stated in Figure 3.

**Figure 5.** Gender and E2 concentrations codetermine GH secretory-burst mode (time-delay to maximal GH release within a burst [overall model $P=0.002$, $R^2=0.45$]. The mode is higher in men than women and increases with E2 concentrations under GHRP-2 drive of feedback escape. A single statistical outlier ($P<0.001$) is marked by a box (*top*). IGF-I represses and E2 heightens basal GH secretion in men and women during feedback escape in the E2/T/GHRH context [overall model $P=0.001$, $R^2=0.50$ with standardized coefficients (std coeff) as noted] (*bottom*).

**Figure 6.** Peptide secretagogue ($P<0.001$) and E2/T treatment ($P<0.001$) but not gender determine approximate entropy (ApEn) of GH secretion patterns in the feedback-enforced setting. The E2 and T effects were significant in women ($P=0.003$) and men ($P=0.020$). During sex-steroid supplementation, peptide effects were GHRP-2>GHRH.
Paired (men/women) columns differ significantly when no alphabetic characters are shared (e.g. \( a \) differs from \( bc \) but not from \( ab \)).

**Appendix  Figure 1**

Triple control of total GH secretion during escape from GH's negative feedback by peptide, gender and sex-steroid treatment. Data are presented as described in the legend of Figure 2.
Table 1. Deconvolution Measures of GH Escape during Negative Feedback

<table>
<thead>
<tr>
<th>Condition</th>
<th>Burst Mass (µg/L)</th>
<th>Pulse No. (per 5.5 h)</th>
<th>Burst Mode (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Placebo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.59 ± 0.24^A</td>
<td>2.2 ± 0.23^A</td>
<td>16 ± 1.2^A</td>
</tr>
<tr>
<td>GHRH</td>
<td>5.7 ± 1.6^B</td>
<td>3.7 ± 0.25^BC</td>
<td>13 ± 0.61^AB</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>5.4 ± 1.4^B</td>
<td>2.8 ± 0.29^AC</td>
<td>16 ± 0.61^A</td>
</tr>
<tr>
<td>Both</td>
<td>12 ± 1.9^BC</td>
<td>3.7 ± 0.29^BC</td>
<td>12 ± 0.68^B</td>
</tr>
<tr>
<td><strong>B. E2/T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>1.4 ± 0.46^AB</td>
<td>2.8 ± 0.29^AC</td>
<td>15 ± 0.8^A</td>
</tr>
<tr>
<td>GHRH</td>
<td>4.8 ± 1.3^B</td>
<td>4.5 ± 0.24^B</td>
<td>14 ± 0.47^AB</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>4.9 ± 1.1^B</td>
<td>3.6 ± 0.33^BC</td>
<td>12 ± 0.88^AB</td>
</tr>
<tr>
<td>Both</td>
<td>13 ± 2.4^C</td>
<td>3.4 ± 0.28^BC</td>
<td>13 ± 0.94^AB</td>
</tr>
</tbody>
</table>

For detailed statistical subanalysis, see Appendix Table 1.

Data are the geometric mean ± SEM for the combined genders (N=25).

Means within columns with no shared alphabetic superscripts differ by Tukey’s *post hoc* test.
Table 2. P Values and Standardized Coefficients of Primary Correlates of Pulsatile GH Escape

<table>
<thead>
<tr>
<th>Condition/Infusion</th>
<th>Overall P [R^2]</th>
<th>Gender (coeff)</th>
<th>E_2 (coeff)</th>
<th>T (coeff)</th>
<th>IGF-I (coeff)</th>
<th>BMI (coeff)</th>
<th>IGFBP-1 (coeff)</th>
<th>IGFBP-3 (coeff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.018</td>
<td>0.007</td>
<td>0.001</td>
<td>0.013</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>[0.684]</td>
<td>(-1.327)</td>
<td>(0.748)</td>
<td>(1.050)</td>
<td>(0.590)</td>
<td>(-0.430)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHRH</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Both</td>
<td>0.004</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.001</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>[0.400]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.691)</td>
<td>(0.459)</td>
</tr>
<tr>
<td>B. E_2/T Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.003</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.022</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>[0.406]</td>
<td></td>
<td></td>
<td></td>
<td>(0.535)</td>
<td></td>
<td>(0.844)</td>
<td></td>
</tr>
<tr>
<td>GHRH</td>
<td>0.026</td>
<td>NS</td>
<td>0.026</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>[0.199]</td>
<td></td>
<td>(0.446)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GHRP-2</td>
<td>0.037</td>
<td>NS</td>
<td>0.037</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>[0.175]</td>
<td></td>
<td>(0.418)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both</td>
<td>0.003</td>
<td>0.003</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>[0.318]</td>
<td>-0.564</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS denotes experiment-wise P>0.05.

Data are P values (and standardized linear-regression coefficients).

Minus for gender denotes women>men (and vice versa).
Regulation of Feedback-Suppressed GH Pulsatility

**Peptide Effects** ($P < 0.001$)

Saline | GHRH | GHRP-2 | Both
---|---|---|---
A | B | B | C

**Gender x Peptide** ($P = 0.377$)

Gender: Men, Women

Saline | GHRH | GHRP-2 | Both
---|---|---|---
A | BC | B | C

**Gender x Treatment** ($P = 0.308$)

Gender: Men, Women

Pulsatile GH Secretion (µg/L/5.5 h)

Treatment: PI, T, PI, E2

Saline | GHRH | GHRP-2 | Both
---|---|---|---
C | AC | A | B

**Treatment x Peptide** ($P < 0.001$)

Treatment: Placebo, Sex Steroid

Saline | GHRH | GHRP-2 | Both
---|---|---|---
A | C | C | D

X:\SEC\Data\1359-04\Figs for Deconv Fdbk Regulation Paper\Figure 1.ppt
Peptide and Treatment Influence Basal GH Secretion

**Peptide Effects (P < 0.001)**

![Peptide Effects Bar Graph]

- Saline
- GHRH
- GHRP-2
- Both

**Gender x Peptide (P < 0.001)**

![Gender x Peptide Bar Graph]

- Men
- Women

**Gender x Treatment (P = 0.053)**

- Men
- Women

**Treatment x Peptide (P = 0.096)**

- Placebo
- Sex Steroid

---

X:\SEC\Data\1359-04\Figs for Deconv Fdbk Regulation Paper\Figure 2.ppt
Feedback-Suppressed Pulsatile GH Secretion

Trivariate $P = 0.003$, $R^2 = 0.477$

- $E_2$ $P = 0.003$
  - Std coeff = 0.58
- BMI $P = 0.004$
  - Std coeff = -0.59
- IGF-I $P = 0.020$
  - Std coeff = 0.43

Placebo Saline

Men (○) Women (●)
**E₂ and Gender Determine Feedback-Inhibited GH Mode**

P = 0.002, R² = 0.45; E₂ P = 0.016, Std Coeff = 0.45; Gender P = 0.001, Std Coeff = -0.66

**IGFBP-1 & Testosterone Predict Basal GH Secretion**

P = 0.001, R² = 0.48; Testosterone (Te) P = 0.007, Std Coeff = 0.52; IGFBP-1 P < 0.001, Std Coeff = 0.78
Joint Impact of E$_2$ and BMI on GH Secretory-Burst Mass

P = 0.011, R$^2$ = 0.34; E$_2$ P = 0.006, Std Coeff = 0.58; BMI P = 0.022, Std Coeff = -0.47

Joint Impact of IGF-I and BMI on Number of GH Bursts

P = 0.005, R$^2$ = 0.38; IGF-I P = 0.002, Std Coeff = 0.66; BMI P = 0.032, Std Coeff = -0.42
Estimates of GH ApEn during Feedback Escape

<table>
<thead>
<tr>
<th>Placebo</th>
<th>Sex Steroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender P = 0.932</td>
<td>women (N = 11)</td>
</tr>
<tr>
<td>Treatment P &lt; 0.001</td>
<td>men (N = 14)</td>
</tr>
<tr>
<td>Peptide P &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

GH ApEn (1, 0.35)

Saline  | GHRH  | GHRP-2 Double
Saline  | GHRH  | GHRP-2 Double

Gender: a
Treatment: ab
Peptide: bc, cd, cde, bc, be

X:\SEC\Data\1359-04\Figs for Deconv Fdbk Regulation Paper\Figure 6.ppt
Appendix Table 1. Primary Statistical Outcomes of Deconvolution Analysis

<table>
<thead>
<tr>
<th></th>
<th>Burst Mass</th>
<th>Pulse No.</th>
<th>Burst Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Model $R^2$</td>
<td>0.69</td>
<td>0.27</td>
<td>0.23</td>
</tr>
<tr>
<td>Overall P</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Covariate P</td>
<td>$&lt;0.001$</td>
<td>0.001</td>
<td>0.086</td>
</tr>
</tbody>
</table>

Main Effects

<table>
<thead>
<tr>
<th></th>
<th>Burst Mass</th>
<th>Pulse No.</th>
<th>Burst Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Gender</td>
<td>$&lt;0.001$</td>
<td>0.403</td>
<td>0.606</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.159</td>
<td>0.019</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Interactions

<table>
<thead>
<tr>
<th></th>
<th>Burst Mass</th>
<th>Pulse No.</th>
<th>Burst Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender x Tx</td>
<td>0.665</td>
<td>0.271</td>
<td>0.551</td>
</tr>
<tr>
<td>Gender x Peptide</td>
<td>0.030</td>
<td>0.107</td>
<td>0.046</td>
</tr>
<tr>
<td>Tx x Peptide</td>
<td>0.001</td>
<td>0.102</td>
<td>0.095</td>
</tr>
<tr>
<td>All 3</td>
<td>0.916</td>
<td>0.388</td>
<td>0.043</td>
</tr>
</tbody>
</table>

A priori Tests

<table>
<thead>
<tr>
<th></th>
<th>Burst Mass</th>
<th>Pulse No.</th>
<th>Burst Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>W Pl vs M Pl</td>
<td>0.017</td>
<td>0.998</td>
<td>0.857</td>
</tr>
<tr>
<td>W Pl vs W E$_2$</td>
<td>0.605</td>
<td>0.093</td>
<td>0.975</td>
</tr>
<tr>
<td>M Pl vs M T</td>
<td>0.881</td>
<td>0.776</td>
<td>0.974</td>
</tr>
<tr>
<td>W E$_2$ vs M T</td>
<td>0.002</td>
<td>0.516</td>
<td>0.999</td>
</tr>
<tr>
<td>GHRH vs GHRP-2</td>
<td>0.999</td>
<td>0.032</td>
<td>0.457</td>
</tr>
<tr>
<td>GHRH vs Dual</td>
<td>$&lt;0.001$</td>
<td>0.389</td>
<td>0.360</td>
</tr>
<tr>
<td>GHRP-2 vs Dual</td>
<td>$&lt;0.001$</td>
<td>0.657</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Data are 3-way ANCOVA outcomes using the placebo/saline results as the covariate (N=11 women, N=15 men). P<0.01 values are underscored.

“Dual” denotes combined GHRH/GHRP-2 infusion.
### Appendix Table 2. Backward Stepwise-Elimination Regression Analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Secondary Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Burst Mass</td>
</tr>
<tr>
<td>A. Placebo</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>E₂(+) / BMI(-)</td>
</tr>
<tr>
<td>GHRH</td>
<td>BMI(-)</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>NS</td>
</tr>
<tr>
<td>Both</td>
<td>NS</td>
</tr>
</tbody>
</table>

B. E₂ / T

<table>
<thead>
<tr>
<th>Condition</th>
<th>Secondary Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Burst Mass</td>
</tr>
<tr>
<td>Saline</td>
<td>NS</td>
</tr>
<tr>
<td>GHRH</td>
<td>BMI(-)</td>
</tr>
<tr>
<td>GHRP-2</td>
<td>NS</td>
</tr>
<tr>
<td>Both</td>
<td>NS</td>
</tr>
</tbody>
</table>

G = gender (+ denotes men > women, - denotes women > men)

E₂ = estradiol

IGF-I = insulin-like growth factor type I

IGFBP-1 = insulin-like growth factor binding protein 1

BMI = body-mass index

NS = not significant

Plus and minus signs denote positive and negative slopes of regression.

Protected P < 0.01 was assumed here.
Regulation of Feedback-Suppressed GH Pulsatility

**Peptide Effects (P < 0.001)**

- Saline
- GHRH
- GHRP-2
- Both

**Gender x Peptide (P = 0.017)**

- Men
- Women

**Gender x Treatment (P = 0.124)**  \( \text{Gender } P < 0.001 \)

- Men
- Women

**Treatment x Peptide (P < 0.001)**  \( \text{Treatment } P < 0.001 \)

- Placebo
- Sex Steroid