A noninvasive measure of negative-feedback strength, approximate entropy, unmasks strong diurnal variations in the regularity of LH secretion

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Liu PY, Iranmanesh A, Keenan DM, Pincus SM, Veldhuis JD. A noninvasive measure of negative-feedback strength, approximate entropy, unmasks strong diurnal variations in the regularity of LH secretion. Am J Physiol Endocrinol Metab 293: E1409–E1415, 2007.—The secretion of anterior-pituitary hormones is subject to negative feedback. Whether negative feedback evolves dynamically over 24 h is not known. Conventional experimental paradigms to test this concept may induce artifacts due to nonphysiological feedback. These limitations might be overcome by a noninvasive methodology to quantify negative feedback continuously over 24 h without disrupting the axis. The present study exploits a recently validated model-free regularity statistic, approximate entropy (ApEn), which monitors feedback changes with high sensitivity and specificity (both >90%; Pincus SM, Hartman ML, Roelfsema F, Thornor MO, Veldhuis JD. Am J Physiol Endocrinol Metab 273: E948–E957, 1999). A time-incremented moving window of ApEn was applied to LH time series obtained by intensive (10-min) blood sampling for four consecutive days (577 successive measurements) in each of eight healthy men. Analyses unveiled marked 24-h variations in ApEn with daily maxima (lowest successive measurements) in each of eight healthy men. The mean difference between maximal and minimal 24-h LH ApEn was 0.348 ± 0.018, which differed by P < 0.001 from all three of randomly shuffled versions of the same LH time series, simulated pulsatile data and assay noise. Analyses artificially limited to 24-h rather than 96-h data yielded reproducibility coefficients of 3.7–9.0% for ApEn maxima and minima. In conclusion, a feedback-sensitive regularity statistic unmasks strong and consistent 24-h rhythmicity of the orderliness of unperturbed pituitary-hormone secretion. These outcomes suggest that ApEn may have general utility in probing dynamic mechanisms mediating feedback in other endocrine systems.

luteinizing hormone; follicle-stimulating hormone; prolactin; male

THE CONCEPTS OF 24-H RHYTHMICITY of hormone secretion and negative feedback control have been known for several decades (5, 22, 26). Nonetheless, a fundamental unresolved question is how the two processes are related. The query is whether 24-h rhythms in hormone output as associated with 24-h cycles in feedback strength. Classical studies of the adrenocorticotropic (ACTH)-glucocorticoid axis demonstrated that corticosteroid feedback in the rat differs in the day and night (1, 2). No comparable data exist for LH, FSH, prolactin, GH, or TSH. In addition, whether negative feedback varies continuously over 24 h is not known in any axis.

Experiments to date have employed pharmacological or surgical ablation of the endogenous feedback signal with or without addback of the feedback effector (1, 9, 23, 29, 31). Although important, ablation-addback models have several potential limitations: 1) multiple feedback signals may operate simultaneously, but are rarely replaced concomitantly; 2) physiological feedback patterns are difficult to reproduce precisely experimentally; 3) pharmacological antagonism of feedback may amplify the secretion of collateral signals; and 4) whether negative feedback varies continuously or changes abruptly across 24 h has not been assessed. For example, both peptides and sex steroids feed back on FSH secretion, making physiological addback of signal(s) difficult after gonadal ablation. In the case of LH secretion, anti-androgen administration inhibits testosterone-mediated negative feedback but elevates estradiol concentrations that also inhibit LH release (24). Both adrenal and gonadal steroids are secreted in pulses that vary in size over 24 h (27, 32), but with rare exceptions both are usually replaced continuously for practical reasons (33). Moreover, most feedback evaluations are made only during a delimited interval in the day or night. To our knowledge, no studies have quantified the time-varying strength of negative feedback continuously over 24 h by nonablative and nonpharmacological means. For these reasons, complementary probes of physiological feedback are needed that can be applied continuously and noninvasively over 24 h.

A regularity statistic, approximate entropy (ApEn), allows one to quantify continuous gradations in feedback signal strength reflected in the orderliness or reproducibility of sub-patterns in serial data (10, 13, 20, 21, 28, 30). ApEn for any given time series is expressed as a single positive number (between zero and the natural logarithm of 10 = 2.30), wherein higher values denote greater relative randomness (less orderliness) associated with decreased negative feedback within the network, and vice versa (14). This validated regularity measure discriminates subtle differences in feedback control with high sensitivity and specificity (both >90%) in seven different experimental paradigms (4, 16, 18, 21, 28, 30, 31), four reductionist mathematical systems, and three integrative physiological models (3, 6, 7, 10, 14). Therefore, we postulated that ApEn would provide a noninvasive means to estimate continuously changing feedback signal strength, thereby offering in complementation of conventional ablation-replacement experiments.

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The present investigation introduces the concept of moving-ApEn estimates to test the hypothesis that unmanipulated negative feedback on LH secretion is subject to 24-h rhythmic control and that this can be unveiled using moving-ApEn. Secondary end points were 24-h rhythms in ApEn of FSH and prolactin. To reduce possible bias, ApEn analyses were applied to 10-min time series collected without interruption over 96 h in each of eight healthy men. In this noninvasive approach, physiological feedback signals and pathways are not deleted or modified.

METHODS

Subjects. Eight men aged 18–36 yr (mean 27 ± 3 yr) participated in the study after providing written, voluntary, informed consent, and the study was approved by the Mayo Clinic’s Institutional Review Board. Participants were healthy, community-dwelling men within 30% of ideal body weight. None had undertaken recent transmeridian travel (within 10 days) or consumed alcohol, caffeine, sedatives, or systemic medications within five biological half-lives. Detailed medical history included any history of infertility, systemic disease, recent weight change (exceeding 2 kg in the preceding 6 wk), androgen therapy, or psychoactive drug use. Outpatient screening was unnecessary in relation to medical history (particularly libido and erectile function), physical examination (including testis size), and fasting morning (0800) biochemical tests of renal, hepatic, hematological, endocrine, and metabolic function.

Protocol. Volunteers were admitted to the General Clinical Research Center (GCRC) the night before blood sampling to allow adaptation to a single private room. The following morning, two catheters were placed in forearm veins in the event of unexpected failure of one. Blood sampling every 10 min commenced at 0800 and continued until 0800 on the morning of the fifth day (i.e., for 96 h). Less than 500 ml of blood was collected from each participant by the use of pediatric blood collection tubes and a miniaturized automated immunoochemiluminometric method, which required only 0.75 ml sampling for each draw. Blood was allowed to clot at room temperature, and sera were frozen at −20°C for later assay of serum LH, FSH, and prolactin (PRL) concentrations.

Volunteers were provided three meals each day during the sampling sessions in the GCRC but were not allowed to snack. Room lights were extinguished at 2300 each evening. Participants remained ambulatory but could not leave the floor, smoke, nap, drink caffeinated beverages, or undertake vigorous exercise during the 4-day sampling period.

Assays. LH concentrations were measured in duplicate by miniaturized automated immunoochemiluminometry (ACS Corning, Bayer, Tarrytown, NY) using the Second International Reference Preparation as standard (31). Intra-assay CVs averaged 5.1% and interassay CVs 6.8%. Procedural sensitivity was 0.05 IU/l. FSH was quantified in the same assay system, wherein mean intra- and interassay CVs were 5.0 and 6.7%, respectively, and sensitivity was 0.2 IU/l. PRL was measured by double-antibody RIA using reagents purchased from Diagnostic Systems Laboratories [Third-Generation DSL-39100, Webster, TX], as reported. Sensitivity was 3 ng/ml and intra- and interassay CVs were 5.4 and 6.9%, respectively. Minimization was possible by increasing indicator and antibody capture concentrations, overnight shaking and robotic pipetting.

Regularity analysis. The ApEn statistic allows one to quantify the degree of regularity or reproducibility of subpatterns within time series (13, 21, 30). ApEn is a model-free, translation- and scale-invariant, asymptotically normally distributed statistic that distinguishes orderliness of data series of length 30 or more points (18, 28, 30). ApEn calibrates an extent of sequential interrelationships, quantifying a continuum that ranges from totally ordered to completely random. ApEn evaluates both dominant and subordinant patterns in data; notably, it will detect changes in underlying episodic behavior not reflected in peak occurrences or amplitudes (20).

Technically, ApEn is defined as the summed logarithmic likelihood that templates (of length m) of patterns in the data that are similar (within r) remain similar (within the same tolerance r) on next (m + 1) incremental comparison (20). ApEn of any given time series is a single nonnegative number, with larger values corresponding to greater apparent process randomness or serial irregularity and smaller values corresponding to more instances of recognizable features or patterns in the data. For the studies discussed herein, ApEn values for all data sets were calculated with widely established parameter values of m = 1 and r = 20% of the SD of each data set. These parameters afford sensitive, specific, valid, and statistically well-replicated regularity measures (18, 28, 30). Normalizing r to each time series SD in this manner gives ApEn translation and scale invariances (15), in that it remains unchanged under uniform process magnification, reduction, or constant shift higher or lower. ApEn is nearly unaffected by noise of magnitude below r, a de facto filter level. ApEn is robust or insensitive to artifacts or outliers: extremely large and small artifacts have small effect on the ApEn calculation, if they occur infrequently. Further technical discussion of mathematical and statistical properties of ApEn can be found elsewhere (17, 19). To develop a more intuitive physiological understanding of the ApEn definition, a multistep description of its typical algorithmic implementation, with figures, is developed in Ref. 17.

To estimate the mean ± SD of random ApEn, each time series was shuffled 1,000 times without replacement. The single authentic (nonrandomized) ApEn for each series was then expressed as a z-score, or number of SDs removed from mean random.

Analytic methods. LH, FSH, and PRL concentrations were initially first-differenced [the i th element of the time series was subtracted from the (i − 1)th element] to ensure that all data were mean stationary. First-differencing is sometimes necessary with shorter time series of pituitary hormone concentrations (11). To calculate regularity locally, the 24-h day was divided into four 6-h windows. Hence, four distinct time-of-day blocks (corresponding to each of the 4 days of sampling) exist for the time period 0800 to 1400 in each subject. These four blocks were sequentially concatenated before ApEn was calculated, and this ApEn number was assigned to the first point of the time period (i.e., 0800). Next, the time-of-day interval 0810 to 1410 was examined identically (and the ApEn value assigned to 0810), and so forth. The result is a series of 144 ApEn numbers quantifying the time evolution of hormone regularity over a 24-h day in each subject (Fig. 1). The concatenation process was designed to ensure reproducibility of the ApEn estimate for each distinct time-of-day period (18, 28, 30).

![Twenty-four Hr Excursions in Pattern Regularity](https://www.ajpendo.org/ajpendo/fig1.png)

Fig. 1. Schema for assessing 24-h cycles in ApEn (approximate entropy), a feedback-sensitive regularity statistic. See METHODS.
Analogous ApEn time series were constructed using 3-, 4-, 5-, and 7-h moving windows to verify consistency and robustness across varying block sizes.

The times of the daily maximum and minimum of ApEn and the absolute daily excursion (maximum minus minimum) of ApEn values were calculated directly for each of the above time series after applying a seven-point moving average. Outcomes were verified using time series constructed of identical block sizes without concatenation. Day-to-day variability across the 4 separate 24-h days was thereby also examined.

**Simulated data.** Two sets of simulated data each containing 30 time series replicates of identical length (corresponding to 10-min sampling over 4 days or 96 h) were constructed. The first set (“white noise”) consisted of random, normally distributed numbers with a mean CV of 8%. The second set (“simulated pulses”) comprised concentration data resulting from simulated hormone secretion at three different pulse frequencies (λ = 14, 18, 22 pulses per 24 h) and four different pulse-time regularity values (γ = 1, 2, 3, 4), assuming a Weibull distribution (8). This model lacks any feedback restraint of orderliness. Analogue ApEn time series were constructed using 3-, 4-, 5-, and 7-h block sizes.

Representative 24-h profiles of LH, FSH, and PRL concentrations on the 1st (lines with circles) and 4th (lines without circles) days of uninterrupted 10-min sampling in a representative subject. Bottom: corresponding 24-h ApEn profiles for LH (left), FSH (middle), and PRL (right) calculated across all 4 days using a moving 6-h time window.

**RESULTS**

Representative 24-h profiles of LH, FSH, and PRL concentrations in one subject are shown in Fig. 2 (top). The reproducibility of 24-h patterns is illustrated by displaying the first and fourth days of sampling. Similar plots were obtained for any combination of the four days of study and for all other times of the daily maximum and minimum of ApEn and the absolute daily excursion (maximum minus minimum) of ApEn values were calculated for each simulated time series, thereby yielding five separate probability histograms against which to compare authentic outcomes. A single probability (P) for each individual across all five window sizes was calculated by averaging. Then, the eight probabilities (one for each participant) were aggregated by Fisher’s method [where \( -2 \log(P) \)] for a \( \chi^2 \) distribution of 2n degrees of freedom], which confers a group P value.

**Table 1. Comparison of hormone ApEn rhythms with simulated data**

<table>
<thead>
<tr>
<th></th>
<th>LH (n = 8)</th>
<th>FSH (n = 8)</th>
<th>PRL (n = 8)</th>
<th>White Noise (n = 30)</th>
<th>Simulated Pulses (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum ApEn*</td>
<td>1.59 ± 0.02**</td>
<td>1.68 ± 0.04**</td>
<td>1.74 ± 0.01**</td>
<td>1.78 ± 0.004**</td>
<td>1.77 ± 0.007**</td>
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<tr>
<td>Time of maximum</td>
<td>1100 ± 1.7</td>
<td>0740 ± 2.3</td>
<td>0700 ± 2.0</td>
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<tr>
<td>Max – Min</td>
<td>0.348 ± 0.018*</td>
<td>0.276 ± 0.046*</td>
<td>0.282 ± 0.026*</td>
<td>0.182 ± 0.006*</td>
<td>0.213 ± 0.008*</td>
</tr>
<tr>
<td>Time gap</td>
<td>100 ± 240 min</td>
<td>110 ± 380 min</td>
<td>10 ± 370 min</td>
<td></td>
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<tr>
<td>Minimum ApEn†</td>
<td>1.24 ± 0.04**</td>
<td>1.40 ± 0.06**</td>
<td>1.45 ± 0.03**</td>
<td>1.60 ± 0.005**</td>
<td>1.55 ± 0.007**</td>
</tr>
<tr>
<td>Time of minimum</td>
<td>0430 ± 1.9</td>
<td>1040 ± 2.5</td>
<td>0800 ± 1.9</td>
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Means ± SE obtained from actual and simulated data. Simulations comprise white noise and simulated pulses. Different superscripts in each row indicate significantly different mean values. Comparison among LH, FSH, and prolactin (PRL) by repeated-measures ANOVA (P > 0.05). Comparison between each hormone and simulated datasets by ANOVA (P < 0.001 for each ANOVA). Times when maximum and minimum approximate entropy (ApEn) occurred are determined by first calculating the mean times for each individual across all windows (3, 4, 5, 6, and 7 h) and then the mean across all 8 individuals. Time gap was calculated by determining the mean of the shortest time gap (in clock time) for each individual across all time windows (3, 4, 5, 6, and 7 h) and then the mean across all 8 individuals. Hence, the time gap is not simply the difference between the group times of maximum and minimum ApEn. *Least orderly; †most orderly.
seven participants (not shown). Nycthemeral evolution of the
regularity (ApEn) of LH, FSH, and PRL concentrations is
depicted for the same participant in Fig. 2 (bottom). Data were
obtained using a moving 6-h ApEn estimation window. Similar
profiles were obtained for each of 3-, 4-, 5-, and 7-h ApEn
windows and for all other participants (not shown).

The means of daily maximum and minimum ApEn estimates
assessed for five time windows (3, 4, 5, 6, and 7 h) in the eight
men are depicted in Table 1. The mean was employed after
analyses indicated that window size did not significantly alter
outcomes (not shown). Next, ApEn is expressed as a z-score
(standardized normal deviate) calculated from the SD and
mean obtained by shuffling each time series 1,000 times to
obtain empirically random distributions. This method of resa-
mpling analysis is illustrated for all five time windows of
moving-ApEn applied to a 4-day LH time series in a single
man (Fig. 3). Resampling in all eight men yielded hormone-
specific probabilities of randomness of \( P < 10^{-5} \) for LH, \( P = 0.003 \) for FSH, and \( P = 0.01 \) for PRL. LH, FSH, and PRL
concentrations were maximally irregular (highest ApEn asso-
ciated with putatively lowest feedback) in the morning near the
time of awakening, viz., clock times 1100, 0740 and 0700,
respectively. Conversely, profiles were maximally regular (pu-
tatively greatest feedback) at 0430 (LH), 1040 (FSH), and
0800 h (PRL). The time gap between maximal and minimal
regularity averaged 100 ± 240 min for LH, -110 ± 380 min for
FSH, and 10 ± 370 min for PRL in the group of eight men
(\( P = \text{NS by hormone type, Kruskal-Wallis test} \)). Minimal
ApEn occurred 370 ± 130 min earlier for LH than for FSH
(\( P = 0.02 \) by signed ranks test). Comparable estimates were

![Fig. 3. Resampling procedure illustrating the random distribution of daily in ApEn (maximum — minimum) values obtained by shuffling one LH concentration time series 1,000 times. Five separate histograms are superimposed, reflecting moving-ApEn window sizes of 3, 4, 5, 6, and 7 h with individual probabilities (\( P \)) and z-scores against chance realizations, thus verifying consistency of inference. Mean \( P \) value and z-score are given. For comparison, the mean observed (nonrandomized) ApEn excursion in the same subject is shown by a vertical arrow and circle (far right).](http://ajpendo.physiology.org/)

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**Table 1**

<table>
<thead>
<tr>
<th>Window Size (h)</th>
<th>Mean ApEn (SD)</th>
<th>Max ApEn (SD)</th>
<th>Min ApEn (SD)</th>
<th>( P ) Value</th>
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<tbody>
<tr>
<td>3</td>
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<td>4</td>
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**Probability Distributions of Random LH ApEn in one Man**

- **Window size and p value**
  - 3 hr \( p = 0.014 \)
  - 4 hr \( p = 0.007 \)
  - 5 hr \( p = 0.003 \)
  - 6 hr \( p = 0.005 \)
  - 7 hr \( p = 0.008 \)

- **P (across all windows) = 0.0054**

- **ApEn difference for non-randomized LH time series**
obtained using the unconcatenated time series (P = NS vs. concatenated by Wilcoxon’s signed ranks test).

The mean times of ApEn nadirs (maximum regularity) are shown in relation to the times (means ± SE) of daily maximum and minimum serum concentrations of LH, FSH, and PRL in Fig. 4. Maximum regularity occurred between the peak and nadir of LH and PRL concentrations. ApEn predicted lowest feedback onto LH at the time of its daily maximum concentration. Day-to-day intraindividual variability in maximum and minimum ApEn was estimated by analyzing each of the 4 days of sampling separately (Table 2). Maximum and minimum ApEn estimates were highly consistent from day to day, as inferred from interday CVs of 3.4 to 9%, which fall within the range of variability estimated for sample collection, processing, and assay (25, 29). However, 24-h estimates of the timing of maximum and minimum ApEn showed high variability (~50%).

As independent assessments of statistical significance, analyses were applied to simulated LH time series (Table 1). There was greater regularity (lower ApEn minimum) in the clinical series than in noise or simulated profiles (each P < 0.001). Greater regularity is consistent with significant feedback control of in vivo hormone release, but not in simulated time series. In addition, differences between maximum and minimum ApEn were significantly greater in authentic LH, FSH, and PRL profiles than either noise or simulated data (each P < 0.001).

Comparisons between ApEn estimates based on 96-h vs. 24-h sampling are shown in Fig. 5. Maximum daily ApEn was consistently underestimated by 1.6 to 4.1 SDs, whereas minimum ApEn was overestimated by 2.0 to 3.5 SDs compared with 96-h data. Although mean estimated times of the daily maxima and minima associated with analyzing 24-h and 96-h data were similar, restricted 24-h estimates yielded large variability (Table 2).

**DISCUSSION**

ApEn, a regularity statistic, quantifies the relative orderliness of sequential data, such as hormonal time series, wherein greater secretory-process orderliness predicts increased negative feedback, and vice versa (12). ApEn has high sensitivity and specificity (both >90%) in detecting feedback differences in ablation-replacement experiments, analytical feedback models, and reductionistic mathematical systems (see introductory section).

The present investigation combined ApEn and an intensive (10-min) and prolonged (4-day) blood sampling regimen to unveil strong and consistent 24-h variations in the regularity of LH secretion. Diurnal rhythms in ApEn were consistent across successive days, as CVs for interdaily ApEn maxima and minima were 5.2–9.0%. Diurnal excursions of LH ApEn exceeded 99.9% statistical confidence intervals determined empirically from assay noise (P < 10^-5), randomly shuffled LH time series (P < 10^-8), and simulated 4-day pulse profiles (P < 10^-10). Comparable but less vivid daily rhythms existed in the secretory orderliness of PRL (P = 0.010) and FSH (P = 0.003). Collectively, these analyses document significant 24-h rhythms in secretory orderliness and thereby presumpively of feedback signal strength. The occurrence of the lowest ApEn values (maximally regular secretory patterns) of LH, FSH, and PRL at 0430, 1040, and 0800 signifies maximal strength of negative feedback at these respective times. This inference is consistent with the evolution of nadir serum LH, FSH, and PRL concentrations in the same time window (0500 to 1300). In particular, nadir LH ApEn values occurred in the early morning, viz., 0430, at the end of the 95% time window of peak serum LH concentrations (0228 to 0456) before the nadir of serum LH concentrations. This sequence would be consis-

### Table 2. Mean intraindividual daily CVs

<table>
<thead>
<tr>
<th></th>
<th>LH</th>
<th>FSH</th>
<th>PRL</th>
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</thead>
<tbody>
<tr>
<td>Minimum ApEn</td>
<td>8.7±2.2</td>
<td>9±1.2</td>
<td>5.2±0.7</td>
</tr>
<tr>
<td>Maximum ApEn</td>
<td>3.7±0.2</td>
<td>3.8±0.9</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>Time of minimum</td>
<td>48±9</td>
<td>45±8</td>
<td>47±6</td>
</tr>
<tr>
<td>Time of maximum</td>
<td>44±4</td>
<td>59±7</td>
<td>44±10</td>
</tr>
</tbody>
</table>

Means ± SE, in %, n = 8 in each group, of coefficients of variation (CVs) showing variation in estimates across the 4 separate days of sampling.

![Fig. 4. Times of daily peak (closed symbols) and nadir (open symbols) LH (squares, top), FSH (diamonds, middle), and PRL (triangles, bottom) concentrations in relation to maximum regularity (lowest ApEn) (diamond-enclosed + sign). Data are means ± SE. Numerical values (right) are the daily (peak – nadir) increments in serum hormone concentrations.](image)

![Fig. 5. Bias introduced by using 24-h rather than 96-h time series to estimate daily ApEn maxima and minima. Data are given for LH, FSH, and PRL in a cohort of 8 men. y-Axis values are z-scores (standard normal deviates) removed from the corresponding mean determined from the 96-h data.](image)
tent with the postulate that antecedent negative feedback contributes to diurnal suppression of LH output. The ApEn minimum for FSH occurred ~3 h later than that for LH (P = 0.02), thus suggesting distinguishable negative-feedback mechanisms for LH and FSH. The association of low ApEn (and thus high feedback) with low serum hormone concentrations is not a technical artifact, because ApEn is a scale-independent measure (17, 28, 30). The present outcomes suggest a new regulatory model, in which diurnal control of feedback intensity modulates nycthemeral rhythms in LH, FSH, and PRL secretion. The degree to which the change in feedback intensity over 24 h is regulated by varying concentrations of the negative-feedback signal and/or by varying responsiveness of selected hypothalamic-pituitary targets of feedback cannot be determined from these data.

Qualifications include the need to corroborate findings in larger cohorts, since the precise timing of maximal regularity varies within and among subjects. In addition, equal and opposite changes in feedback and feedforward strength would not be detectable readily using ApEn analysis. This unexplored possibility might be assessed by measuring both the feedback and feedforward signal simultaneously (11).

In summary, quantification of secretory-process regularity in healthy young men by use of a moving-average ApEn statistic and intensive (10-min), extended (4-day) hormone sampling demonstrates prominent daily cycles in putative feedback control of LH (and to a lesser degree FSH and prolactin) secretion. These data support the concept that diurnal variations in feedback intensity modulate 24-h rhythms in pituitary hormone secretion. Further studies will be required to assess the generality of this concept and elucidate the precise mechanistic basis for time-varying feedback control.

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