Analysis of bidirectional pattern synchrony of concentration-secretion pairs: implementation in the human testicular and adrenal axes


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have corroborated relative androgen depletion in aging men. Other epidemiological data have correlated hypoandrogenemia with sarcopenia, osteopenia, reduced physical stamina, sexual dysfunction, impaired quality of life, and (possibly) depressive mood and cognitive impairment (46). Recent interventional studies suggest that reduced testosterone availability may be relevant to frailty in aging men (18, 36). In parallel with therapeutic notions, mechanistic investigations are needed to determine why aging is associated with hypoandrogenemia. Few studies have appraised the effect of aging on nonlinear interactions, which are expected from time-delayed asymptotic dose-response connections among gonadotropin-releasing hormone (GnRH), LH, and testosterone. Recent developments of regulatory statistics and their validation in mathematical, laboratory-based, and clinical contexts may address this impasse (15, 22, 26).

Aging and persistent stress also appear to force subtle adaptations in integrative control of the hypothalamo-pituitary-adrenal (HPA) axis (34). In large population-based studies, late-day cortisol concentrations rise consistently with age, whereas single morning values are variable (1, 3, 4, 13, 17, 38, 39, 51). Feedback analyses based on acute administration of synthetic and natural glucocorticoids suggest diminished inhibitory efficacy in older individuals, especially in women. These data point to subtle age and gender differences in feedback regulation. Such changes may be detrimental in the long run, since the stress-responsive corticotropic axis is vital for maintenance of glucose and salt balance, blood pressure, well-being, and normal longevity. Chronic over- or underexposure to corticosteroids impairs glucose homeostasis and central nervous system, bone, muscle, and cardiovascular function, illustrating that tight regulation of this axis is required (35). Although basic feedback and feedback linkages are well described, the relative stability and symmetry of in vivo adrenocorticotropic hormone (ACTH)-cortisol feedback and cortisol-ACTH feedback links are not known in any species. The analytic challenge is to achieve accurate quantification of partially coupled time-evolving signals in the HPA axis without disruption of endogenous interactions.

One broad class of quantitative nonlinear, model-invariant tools to assess time-varying coordination between interlinked hormones is the approximate entropy (ApEn) statistic (22). The univariate ApEn measure discriminates relative serial regularity or recurring orderliness within different complex patterns.

AGING IS MARKED BY A 30–50% decline in systemic testosterone concentrations in healthy community-dwelling men, as recognized nearly 50 yr ago (11, 46). Subsequent longitudinal (5, 9, 20) and population-based cross-sectional (2, 7, 19, 37) studies

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such as pulsatile neurohormonal output. Two relevant features of this statistical perspective are 1) applicability to the un fused and unstressed state without disruption of ongoing time-adaptive axis interactions and 2) validity independent of the absolute scale of measurement. The corresponding cross-ApEn statistic quantifies the relative synchrony of two interlinked signals, such as paired sequential hormone measurements in the gonadal axis (30). To date, only the feedforward “direction” of coupling has been investigated by this means in neurohormonal systems, i.e., the LH drive of testosterone and the ACTH drive of cortisol concentrations (30, 33). Therefore, the present methodology provides a model-free and scale-invariant strategy to estimate two-signal interactions in a closed and coupled neurohormone system without requiring external interventions.

Illustratively, we examine the hypotheses that 1) both feed forward and feedback coordinations of GnRH-LH-testosterone are detectably disrupted in aging men, as monitored in the absence of external interventions, and 2) unmodified in vivo ACTH-cortisol feedforward and cortisol-ACTH feedback linkages in the aging adult are relatively stable and symmetrical.

METHODS

Clinical sampling protocol to assess the male gonadal axis. Eleven young (21–31 yr old) and eight older (62–74 yr old) men were enrolled, after they provided voluntary written, informed consent, as approved by the Institutional Review Board. Participants were healthy community-dwelling men within 20% of ideal body weight who had not undertaken transmeridian travel or consumed alcohol, caffeine, prescribed medications, or recreational drugs. Detailed medical inventory excluded a history of infertility, systemic disease, recent weight change, hormonal therapy, or psychoactive drug use. Physical examination (including testis size, libido, and potency) and morning (0800) fasting biochemical tests of renal, hepatic, hematological, and metabolic functions (fasting plasma glucose and thyroid functions) were within normal limits. Men were admitted to a single room in the General Clinical Research Center for two consecutive nights, with the first night to allow adaptation to the unit. On the second night, subjects underwent blood sampling every 2.5 min starting at 2300, when lights were turned off, until the subject awakened, for an average duration of 7 h. Blood was withdrawn via heparin-coated long lines by a phlebotomist working in an adjacent room to prevent sleep disturbance. Further subject details are provided elsewhere (21).

Clinical sampling protocol to assess the adrenal axis. Thirty-five normal subjects (17 women and 18 men) aged 26–77 yr (mean of 44 yr) were enrolled after they provided written, informed consent, as approved by the responsible ethics committee. Volunteers were healthy community-dwelling men and women within 20% of ideal body weight who had not received glucocorticoids or any prescribed medications within the preceding 3 mo and had not undertaken transmeridian travel within the preceding 2 wk. Detailed medical inventory excluded a history of psychiatric illness (including depression), systemic disease, recent weight change, hormonal therapy, and alcohol or psychoactive drug abuse. Physical examination and morning fasting biochemical tests of renal, hepatic, hematological, and metabolic functions (fasting plasma glucose and thyroid functions) were within normal limits. Participants were hospitalized the evening before the sampling studies, and an indwelling intravenous cannula was inserted into a large forearm vein in the morning. Premenopausal women were studied in the early follicular phase of the menstrual cycle. Blood samples were withdrawn every 10 min for 24 h starting at 0900. Subjects were allowed to ambulate freely but were not allowed to sleep during the day. Lights were turned off between 2200 and 2400, depending on individual sleep habits. Further subject details are provided elsewhere (33).

Assays. Blood samples were allowed to clot at room temperature, and sera were frozen at −20°C. LH concentrations were assayed in duplicate by specific LH β-subunit-directed two-site monoclonal immunoradiometric assay (IRMA) (Nichols Laboratories, San Juan Capistrano, CA) (21, 49). The sensitivity of the LH assay is 0.2 IU/l (First International Reference Preparation), and there is <0.03% cross-reactivity with free α-FSH or TSH (42, 49, 50, 52). The intraand interassay coefficients of variation (CVs) are <8% and <10%, receptively. Total testosterone concentrations were measured in duplicate by solid-phase RIA (Diagnostic Products, Los Angeles, CA, and Diagnostic laboratory Systems, Webster, TX). Assay sensitivity is 20 ng/dl, and intra- and interassay imprecisions are <7.5% and <10%, respectively. Dose-dependent intra-assay variance was estimated as power functions from the entire set of sample replicates in any one subject.

ACTH samples were collected on ice in chilled EDTA-containing siliconized glass tubes, and cortisol samples were collected in heparinized tubes. Blood was centrifuged at 4°C, and the plasma was separated within 30 min and then frozen at −20°C for later analyses. ACTH concentrations were assayed in duplicate by immunoradiometric assay (Nichols Laboratories) (33). The sensitivity of the ACTH assay is 3.0 ng/l. There is <0.1% cross-reactivity with α-MSH, LFSH, TSH, growth hormone, and prolactin. The intra- and interassay CVs are <6% and <7.5%, respectively. Plasma concentrations of total cortisol were measured by RIA (Sorin Biomedica, Milan, Italy). Assay sensitivity is 25 mmol/l, and the interassay CV is <4%.

Calculation of secretion. LH secretion was deconvolved from measured LH concentrations, using a secretion-waveform-indepen- dendent method (45, 48) that utilizes population-defined biexponential elimination kinetics. ACTH, testosterone, and cortisol secretion rates were deconvolved analogously. Half-lives (in min) assumed were 18, 90, and 0.63 (rapid, slow, and proportion of slow/total) for LH; 3.5, 14, and 0.67 for ACTH; 4.9, 48, and 0.18 for testosterone; and 3.8, 66, and 0.67 for cortisol (12, 14–16, 41). These half-lives have been previously validated. Furthermore, half-life changes within the degree of variability recognized in healthy populations do not strongly influence ApEn (47).

Cross-ApEn statistic. The ApEn statistic was developed to quantify the degree of irregularity, or disorderliness, of a single time series (22, 30). Technically, ApEn quantifies 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 the next incremental comparison (29), as validated elsewhere (27). This method has been extensively described (26, 27, 29, 31). The ApEn provides a single nonnegative number, which is an ensemble estimate of relative process randomness, wherein larger ApEn values denote greater irregularity (44). It has been widely applied to similar hormonal data (25, 30, 33, 43).

Analogously, the cross-ApEn quantifies joint pattern synchrony between two simultaneously measured time series (24, 30, 31, 33); the APPENDIX contains the formal mathematical definition of cross-ApEn. In this context, synchrony refers to pattern similarity, not synchrony in time, wherein patterns in one series appear (within a certain tolerance) in the other series. In the present time series, we calculated cross-ApEn values with r = 20% of the SD of the individual subject’s hormone time series and m = 1; for this, we hence use the designation X-ApEn(L.20%). This parameter set affords sensitive, valid, and statistically well-replicated ApEn (6, 10, 25, 28) and cross-ApEn (30, 40, 43) metrics, which are stable to increasing experimental error or sampling frequency (28, 47) in hormone time series assessments of this length. These aforementioned statistical evaluations include a variety of both broad-based theoretical analyses and numerous diverse applications, under (approximate) mean stationarity. In particular, these evaluations establish that one SD of both ApEn and X-ApEn is ≤0.06 for virtually all processes analyzed for the data lengths studied.
LH-testosterone (not shown). The intent of such pairing is to more closely mimic in vivo coupling. Reverse X-ApEn is calculated analogously. Differential X-ApEn is calculated as the difference, i.e., forward minus X-ApEn, in any given paired series. Hence, if differential X-ApEn is positive, it indicates that forward X-ApEn exceeds reverse X-ApEn. To allow comparison, concentration-concentration correlation and ACTH secretion (feedback, right) time series. Concentration-secretion but not concentration-concentration comparisons disclosed more synchronous feedback (lower X-ApEn) than feedforward in both age groups. In both analy-
Aging disrupts the synchrony of both LH-stimulated testosterone patterns and testosterone-inhibited LH release patterns (DISCUSSION).

Figure 3, top, summarizes differential X-ApEn values defined as the difference between reverse and forward X-ApEn in each subject, based on concentration-concentration (left) and concentration-secretion (right) LH-testosterone pairs. Age did not influence relative feedback/feedforward symmetry; that is, differential X-ApEn did not differ between young and old ($P > 0.05$), suggesting that feedforward and feedback synchrony, albeit reduced in aging, does not differ in relative degree of reduction. We also examined whether the mean within-subject difference between reverse and forward X-ApEn values was significantly different from zero in each age cohort. In young men, borderline asymmetry was inferred in differential X-ApEn at concentration-concentration level ($P = 0.047$). In contrast, differential X-ApEn estimated from concentration-secretion pairs disclosed a marked difference in symmetry (Fig. 3, right, $P < 0.0001$). In both young and older men, reverse X-ApEn was less than forward X-ApEn, indicating greater pattern regularity of feedback (testosterone’s inhibition of LH secretion) than feedforward (LH’s stimulation of testosterone secretion). Figure 3, bottom, shows the regression of reverse X-ApEn on forward X-ApEn, thereby illustrating strong asymmetry for concentration-secretion but not concentration-concentration relationships.

Figure 4 summarizes forward (top) and reverse (bottom) X-ApEn estimates based on ACTH-cortisol and cortisol-ACTH concentration-concentration (left) and concentration-secretion (right) time series. These data show that feedforward (ACTH effect on cortisol) and feedback (cortisol effect on ACTH) linkages are comparable in men and women.

Figure 5 presents differential X-ApEn (top) and the regression of reverse X-ApEn on forward X-ApEn (bottom) calculated from concentration-secretion (left) and concentration-concentration (right) ACTH-cortisol and cortisol-ACTH data pairs. As evident from Fig. 5, top, gender did not influence relative feedback/feedforward symmetry; that is, differential X-ApEn did not differ according to gender ($P > 0.05$). Mean differential X-ApEn calculated in concentration-secretion data pairs was significantly negative in men and women ($P < 0.05$ for each, Fig. 5, right) but not in concentration-concentration hormone pairs ($P > 0.05$, Fig. 5, left). For analysis of the combined cohort, X-ApEn differences were $-0.17 \pm 0.04$ (concentration-secretion, $P < 0.0003$) and $0.009 \pm 0.03$ (concentration-concentration, $P = 0.08$). This indicates greater pattern synchrony in the feedforward direction than in the feedback direction for concentration-secretion data pairs.

Linear regression disclosed a small ($R^2 = 0.16$) but significant ($P = 0.017$) positive correlation of cortisol concentration-ACTH concentration X-ApEn on age (data not shown). No other significant correlations emerged (see caveats DISCUSSION).

Power and inverse exponential regression of X-ApEn on age did not improve model fits (data not shown).

**DISCUSSION**

The present analyses highlight several insights gained by application of forward, reverse, and differential X-ApEn to physiologically pertinent paired concentration-secretion signals in two separate prototypical coupled neuroendocrine systems and shows that 1) concentration-secretion signals are more effective than concentration-concentration signals in unveiling asymmetries between feedforward and feedback cou-
pling and 2) both feedforward and feedback coupling should be assessed. This work thereby extends previously presented concentration-concentration forward X-ApEn analyses (30, 33).

We initially tested the impact of age stratum in healthy men on quantitative estimates of the in vivo feedforward (forward) action of LH on testicular Leydig cell testosterone production and the reciprocal feedback (reverse) action of testosterone on hypothalamopituitary inhibition of LH secretion. The resulting contrasts between healthy young and older men support the notion that aging is marked by significant erosion of both LH-testosterone feedforward and testosterone-LH feedback synchrony. Reduced feedback synchrony between testosterone and LH in older men complements previous reports that assessed only feedforward linkages. The age-associated reduction in two-hormone synchrony was comparable in the forward and reverse directions in that differential X-ApEn values did not differ significantly in young and older men. Unexpectedly, in both age strata, forward X-ApEn was greater than reverse X-ApEn (because differential X-ApEn was significantly positive and nonzero). Because lower X-ApEn denotes greater synchrony, this outcome means that testosterone concentration-LH secretion feedback linkages are more tightly coordinated than those of LH concentration-testosterone secretion feedforward at any age.

Analyses in the corticotropic axis revealed significantly greater feedforward than feedback synchrony of ACTH concentration-cortisol secretion patterns. More orderly ACTH concentration drive of cortisol secretion (than concentration inhibition of ACTH secretion) was independent of gender and age in the cohort of 35 adults studied here. However, an important caveat is that only three subjects were 60–77 yr old, and no subjects were 18–25 yr old. Accordingly, the present outcomes do not exclude gender- and/or age-related differences in stimulatory vs. inhibitory coupling in the extreme age ranges.

The accompanying comparisons highlight the importance of assessing both forward and reverse linkages between physiologically relevant signal pairs. In addition, detection of greater feedback than feedforward coupling between LH and testosterone requires pertinent X-ApEn formulation of a concentration input driving (LH on testosterone) or inhibiting (testosterone on LH) secretion. The asymmetry of this reciprocal linkage was highly significant ($P < 10^{-4}$) but only marginally ($P = 0.047$) detectable in young men, according to concentration-concentration data. Similarly, for ACTH and cortisol coupling, X-ApEn contrasts at the concentration-concentration level were less analytically sensitive to detecting asymmetry of reciprocal two-hormone signaling. These distinctions underscore the utility of quantitatively relevant concentration-secretion linkages by initial deconvolution estimates of secretion.

The present outcomes must be distinguished conceptually from measurements of individual ACTH or cortisol concentrations or production rates, which have disclosed both age (3, 4, 17, 38, 39, 51) and gender (8, 17, 32, 38) disparities. One difference is that synchrony between input (effector concentration) and output (secretion response) patterns as assessed here is scale invariant. Second, the notion of X-ApEn quantitation of directional coupling between two signals is model free (METHODS). Third, analyses are applied without experimental perturbation of any component of the HPA axis. The absence...
of significant external stress is supported by normal circadian variations of ACTH and cortisol concentration reported earlier (33) in this healthy adult cohort. Fourth, feedforward/feedback asymmetry of ACTH-cortisol and cortisol-ACTH synchrony can be assessed on a within-subject basis. This point obviates potential loss of statistical power associated with biological variability among individuals.

In summary, we highlight pairwise analyses of the relationship between an input concentration signal and a biologically linked output secretion signal using a model and scale-independent pattern-sensitive measure of feedforward and feedback synchrony. This analytical strategy in the male gonadal axis identifies disruption of both feedforward and feedback linkages in aging individuals, shows directionally comparable loss of synchrony, and reveals that feedback coupling patterns are more reproducible than those of feedforward in both age cohorts. In contrast, healthy adults maintain greater joint synchrony of ACTH concentration-dependent feedforward drive of cortisol secretion than of cortisol concentration-dependent feedback repression of ACTH secretion independently of age and gender.

Further elucidation of the mechanisms by which feedforward and feedback linkages change with age might involve development of a theoretical model of the entire GnRH-LH-testosterone or hypothalamic-ACTH-cortisol systems, including individual hormone kinetics, secretion rates, binding to carrier proteins, and properties of feedforward and feedback interfaces. Computer modeling of these networks and estimation of parameter values from empirical data should aid understanding.

Perspectives

This work highlights application of a nonlinear method to assess pattern synchrony in physiologically coupled concentration-secretion feedforward and feedback hormone pairs. The present analytic strategy should be useful in dissecting mechanistic control in other neuroendocrine systems with strong connectivity of signals, such as FSH-estradiol, TSH-thyroxine and growth hormone-IGF-I or even more broadly may be useful as a general methodological paradigm for network analyses beyond the endocrine setting.

APPENDIX

Definition of Cross-ApEn

To quantify asynchrony (conditional irregularity), we utilized cross-ApEn (introduced in Ref. 31, definition 5). The precise definition, given next, is thematically similar to that for ApEn.

For the definition of Cross-ApEn, let \( u = [u(1), u(2), \ldots, u(N)] \) and \( v = [v(1), v(2), \ldots, v(N)] \) be two length \( N \) sequences; \( m \) and \( r \) are fixed-input parameters. We then form vector sequences \( x(i) = [u(i), u(i+1), \ldots, u(i+m-1)] \) and \( y(j) = [v(j), v(j+1), \ldots, v(j+m-1)] \) from \( u \) and \( v \), respectively. For each \( i \leq N - m + 1 \), we set

\[
C_m^r(v | u) = (\text{number of } j \leq N - m + 1 \text{ such that } d(x(i), y(j)) \leq r) / (N - m + 1),
\]

where \( d(x(i), y(j)) = \max_{k=1,2,\ldots,m} | u(i+k-1) - v(j+k-1) | \), i.e., the maximum difference in their respective scalar components. The \( C_m^r(v | u) \) measure represents, within a tolerance \( r \), the regularity, or frequency, of \((v-)\) patterns similar to a given \((u-)\) pattern of window length \( m \).

We then define \( \Phi^m(r | | u) \) as the average value of \( \ln C_m^r(v | u) \) and finally define

\[
\text{cross-ApEn}(m, r, N)(v | u) = \Phi^m(r | | u) - \Phi^{m+1}(r | | u)
\]

For the present study, we applied cross-ApEn with \( m = 1 \) and \( r = 0.2 \) to standardized \( u-v \) time-series data; i.e., for each subject, we applied cross-ApEn(1, 0.2) to the \([u^m(i), v^m(i)]\) series, where \( u^m(i) = [u(i) - \text{mean } u]/\text{SD } u \) and \( v^m(i) = [v(i) - \text{mean } v]/\text{SD } v \). This standardization, in conjunction with the choice of \( m \) and \( r \), ensures good replicability properties for cross-ApEn for the data lengths studied.

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