Appendix

ACTH, CRH and AVP secretion rates and cortisol concentrations were measured by intensive sampling of pituitary venous (peptides) and internal-jugular (cortisol) blood in normal horses. Three horses were studied before and during metyrapone administration and 3 others before and during insulin-induced hypoglycemia. In each horse, ACTH, CRH and AVP secretion (flow x concentration) and cortisol concentrations were observed for 0.5 h before and approximately 2.5 hr after the onset of a stimulus. In 2 of the insulin-induced hypoglycemia horses, the sampling rate was every 1 min, and in the other 4 horses it was every 30 sec.

For hypothalamic CRH (C) and AVP (V) secretion, assume that burst-like release is described by two terms: (i) a waveform (ψ) or instantaneous (unit-area normalized) rate of secretion over time, $\psi(\cdot)$; and (ii) a mass (M) released per unit distribution volume (29). The burst times for CRH and AVP were estimated by a methodology that first removes any trends and then creates an exhaustive collection of candidate burst-time sets using a smoothing procedure (a nonlinear version of the diffusion equation) (30). The pulsatile secretion model represents secretory-burst mass as a linear function of the preceding interpulse interval plus a random effect. The model is fitted for each candidate pulse set, and the optimal pulse set is then chosen via an Akaike Information Criterion (AIC) that penalizes a larger number of pulses. The resulting CRH and AVP bursts times are denoted as, respectively, $(T_C^{(1)}, T_C^{(2)}, \cdots, T_C^{(m_C)})$ and $(T_V^{(1)}, T_V^{(2)}, \cdots, T_V^{(m_V)})$. Secretory burst mass is assumed to be a logistic function of the cortisol concentration (slow negative feedback), the rate of change in cortisol concentrations (rapid negative feedback), and negative autofeedback by each neuropeptide. In order to accommodate

physiological pulse-by-pulse variations in burst mass, random effects are allowed in pulse-by-pulse dose-response efficacy. Random effects, $(A_C^{(1)}, A_C^{(2)}, \cdots, A_C^{(m_C)})$ and $(A_V^{(1)}, A_V^{(2)}, \cdots, A_V^{(m_V)})$, are assumed to be independently and identically distributed (IID) normal with mean zero and standard deviations, σ_{A_C} and σ_{A_V}. Thus, the k-th CRH and j-th AVP burst masses under triple feedback by CORT (mean cortisol), DCORT (derivative cortisol) and the peptide itself are given as:

$$M_C^{(k)} = \eta_C^{(2)} + \frac{Eff_C + A_C^{(k)}}{[1+\exp\{-(\eta_C^{(0)} + \eta_C^{(1)} \times CORT_C^{(k)}][1+\exp\{-(\gamma_C^{(0)} + \gamma_C^{(1)} \times DCORT_C^{(k)}][1+\exp\{-(\phi_C^{(0)} + \phi_C^{(1)} \times CRH_C^{(k)}]} \quad (1)$$

$$M_V^{(j)} = \eta_V^{(2)} + \frac{Eff_V + A_V^{(j)}}{[1+\exp\{-(\eta_V^{(0)} + \eta_V^{(1)} \times CORT_V^{(k)}][1+\exp\{-(\gamma_V^{(0)} + \gamma_V^{(1)} \times DCORT_V^{(j)}][1+\exp\{-(\phi_V^{(0)} + \phi_V^{(1)} \times AVP_V^{(j)}]} \quad (2)$$

with $\eta^{(1)} \geq 0, \gamma^{(1)} \leq 0, \varphi^{(1)} \leq 0$. The inputs into the burst mass dose-response: cortisol, cortisol derivative, and CRH and AVP, are their mean values over the respective interpulse interval. For example, $CORT_C^{(k)}$ is the mean cortisol concentration over the CRH interpulse interval $(T_C^{(k)}, T_C^{(k+1)})$. The CRH and AVP waveform functions (burst shapes) are defined by the generalized Gamma probability density:

$$\psi_r(s) \propto s^{\beta_r^{(1)}\beta_r^{(3)}-1} e^{-(s/\beta_r^{(2)})^{\beta_r^{(3)}}}, r = CRH, AVP. \quad (3)$$

The resulting CRH (C) and AVP (V) secretion rates are thus given as:

$$Z_C(t_i) = \beta_C^{(0)} + \sum_{T_C^{(k)} \leq t_i} M_C^{(k)} \times \psi_C(t_i - T_C^{(k)}) \quad (4)$$

$$Z_V(t_i) = \beta_V^{(0)} + \sum_{T_V^{(j)} \leq t_i} M_V^{(j)} \times \psi_V(t_i - T_V^{(j)}). \quad (5)$$

The observed CRH and AVP secretion rates, $Y_{C,i}$ and $Y_{V,i}$, with measurement error, are:

$$Y_{C,i} = Z_C(t_i) + e_{C,i} \quad \text{and} \quad Y_{V,i} = Z_V(t_i) + e_{V,i}\text{, i=1,…,n,} \tag{6}$$

where the error terms: $e_{C,i}$ and $e_{V,i}$ are assumed to be IID Normal with mean zero and standard deviations, σ_{e_C} and σ_{e_V}. Under the above assumptions, a Gaussian likelihood function can be written for the observed CRH secretion rates and for the observed AVP secretion rates. Maximum-likelihood estimates (MLE) of the parameters can be obtained. The random effects are then replaced by their conditional expectation, given the data, and evaluated at the MLE, *e.g.*,

$$\hat{A}_C^{(k)} = E_{\hat{\theta}_C}(A_C^{(k)} \mid Y_{C,1}, Y_{C,2}, \ldots, Y_{C,n}). \tag{7}$$

This methodology is employed to obtain secretion-profile fits and estimated dose-response functions for CRH and AVP.

In order to capture the joint dynamic nature of cortisol's feedback onto ACTH secretion via inhibition of both hypothalamic CRH and AVP secretion (amount) and pituitary CRH and AVP feedforward on ACTH secretion (action), successive windows of data length 40 min were examined. Windows were shifted through the full time series by 10 min each. Let $\{(w_r^{(1)}, w_r^{(2)}), r = 1,2,\cdots,K\}$ denote the windows, and let $Cort_{Wndw,r}$, r=1,…,K, denote the cortisol concentration at the start of the K windows. Dual peptide-driven (jointly determined) ACTH secretion rates, one for each window, are:

$$Z_A^{(r)}(t_i) = \lambda_A^{(r,0)} + \frac{Eff_A^{(r)}}{[1+\exp\{-(\lambda_A^{(r,1)} + \lambda_A^{(r,2)} \times AVP(t_i)\}][1+\exp\{-(\lambda_A^{(r,3)} + \lambda_A^{(r,4)} \times CRH(t_i)\}]},$$

$$w_r^{(1)} \le t_i \le w_r^{(2)} \tag{8}$$

To assess the individual effects and the synergism between AVP and CRH, one defines the dose-response for ACTH secretion on CRH alone (AVP set equal to zero:

V=0) and that of ACTH secretion on AVP alone (CRH set equal to zero: C=0):

$$Z_{A,V=0}^{(r)}(t_i)=\lambda_A^{(r,0)}+\frac{Eff_A^{(r)}}{[1+\exp\{-(\lambda_A^{(r,1)}+\lambda_A^{(r,2)}\times 0]\,[1+\exp\{-(\lambda_A^{(r,3)}+\lambda_A^{(r,4)}\times CRH\,(t_i)]},$$

$$w_r^{(1)}\le t_i\le w_r^{(2)} \qquad (9)$$

$$Z_{A,C=0}^{(r)}(t_i)=\lambda_A^{(r,0)}+\frac{Eff_A^{(r)}}{[1+\exp\{-(\lambda_A^{(r,1)}+\lambda_A^{(r,2)}\times AVP(t_i)]\,[1+\exp\{-(\lambda_A^{(r,3)}+\lambda_A^{(r,4)}\times 0]},$$

$$w_r^{(1)}\le t_i\le w_r^{(2)} \qquad (10)$$

The ACTH secretion rates $Y_{A,i}^{(r)}$, within the r-th window, are assumed to be given as:

$$Y_{A,i}^{(r)}=Z_A^{(r)}(t_i)+e_{A,i},\quad w_r^{(1)}\le t_i\le w_r^{(2)},\ \text{r=1,…,K,} \qquad (11)$$

where $e_{A,i}$ are IID normal with mean zero and standard deviation, σ_{e_A}. A Gaussian likelihood can be constructed for ACTH secretion, conditional on the observed CRH and AVP data. Maximum-likelihood estimates of the parameters can be obtained, and the resulting estimated two-dimensional dose-response surfaces calculated for each window using equation (8).

Observed ACTH, CRH and AVP secretion can be plotted (Figure 2) against the starting values of cortisol for each window. Cortisol-dependent inhibition is apparent after metyrapone administration over the full time and during hypoglycemia after the first hour. A goal was to partition cortisol's inhibition into its hypothalamic effects and its pituitary effects, and in the process quantify the degree of CRH/AVP synergism. To illustrate dynamic changes, denote the 84th percentile value (mean plus one standard deviation in a one-tailed model) of CRH and AVP secretion rate values for each window as $CRH_{Wndw,r}$ and $AVP_{Wndw,r}$, r=1,…,K.

Consequently, for each window (r), evaluating the dose-response for ACTH secretion [equation (8)] at the values: $AVP_{Wndw,r}$ and $CRH_{Wndw,r}$, one obtains a value $ACTH^{(C,V)}_{wndw,r}$. Using equations (9)-(10), one obtains values for ACTH secretion based upon $CRH_{Wndw,r}$ alone (where AVP=0), and based upon $AVP_{Wndw,r}$ alone (where CRH=0). These two values we denote as: $ACTH^{(C)}_{wndw,r}$ and $ACTH^{(V)}_{wndw,r}$. Such system values, created from the parameter estimates, capture the underlying dynamics more strongly than the mean data:

(a) full pathway: $(CORT_{Wndw,r}, AVP_{Wndw,r}, CRH_{Wndw,r}, ACTH^{(C,V)}_{wndw,r})$,

(b) CRH alone: $(CORT_{Wndw,r}, CRH_{Wndw,r}, ACTH^{(C)}_{wndw,r})$, r=1,…,K

(c) AVP alone: $(CORT_{Wndw,r}, AVP_{Wndw,r}, , ACTH^{(V)}_{wndw,r})$. (12)

One plots each of these ACTH responses ((a)-(c)) and analyzes the strengths of the relationships, *e.g.*, by linear regression. This strategy has the advantage of simplicity.

Synergism was defined (and calculated) in three alternative forms: first, as the difference between jointly determined ACTH secretion [Equation 12a], and the larger of that induced by either peptide alone [Equations 12b, 12c]; secondly, as the difference between jointly determined ACTH secretion [Equation 12a], and the sum of those induced by each peptide alone [Equations 12b, 12c]; and, thirdly (a potentiation synergy), as the difference between the joint potency effect and the sum of the ACTH effects of CRH and AVP at their potencies [utilizing Equations 8-10]. The relationship between ACTH and cortisol secretion, which is mediated via both hypothalamic and

pituitary inhibition, can be assessed through the changing nature of ACTH, CRH and AVP secretion rates with respect to changing concentrations of $CORT_{Wndw,r}$ over successive windows.

To illustrate aggregate inferences for the 3 horses given metyrapone and 3 others subjected to hypoglycemia, the full cortisol dynamic range observed in each animal was divided into thirds. Data from all 3 horses in each cohort then were analyzed together for each cortisol stratum (0-33%, 34-67%, 68-100%). In this approach, data are segmented by relative cortisol concentrations rather than by 40-min time windows. One thereby constructs the logistic dose-response surfaces linking CRH → ACTH and AVP → CRH at each cortisol stratum.