Studies of the Secretion of Corticotropin-releasing Factor and Arginine Vasopressin into the Hypophysial-Portal Circulation of the Conscious Sheep

II. The Central Noradrenergic and Neuropeptide Y Pathways Cause Immediate and Prolonged Hypothalamic-Pituitary-Adrenal Activation. Potential Involvement in the Pseudo-Cushing's Syndrome of Endogenous Depression and Anorexia Nervosa

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Abstract

Studies were performed to determine the effects of intracerebroventricular norepinephrine (NE) or neuropeptide Y (NPY) on the ovine hypothalamic-pituitary-adrenal (HPA) axis. NE (50 μg) increased mean hypophysial-portal corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) levels (1 h, 1.3- and 2.9-fold; 4 h, 2.2- and 5.7-fold) and caused acute and sustained increases in mean plasma ACTH and cortisol. NPY (50 μg) also increased mean CRF and AVP levels (1 h, 1.4- and 4.2-fold; 4 h, 1.1- and 1.9-fold), increased pituitary-adrenal activity at 1 h, and caused ACTH hypersecretion at 4 h. When added to cultured ovine anterior pituitary cells, NPY neither increased basal ACTH release nor augmented CRF- or AVP-induced ACTH release. We conclude that: (*a*) activation of either the central noradrenergic or NPY pathways causes an acute and sustained stimulation of the ovine HPA axis; (*b*) such activation increases the AVP/CRF ratio, suggesting a dominant role for AVP in the ovine stress response; and (*c*) the central noradrenergic or NPY systems may cause sustained HPA activation by attenuating or disrupting the glucocorticoid negative feedback on those brain areas concerned with regulation of the HPA axis. The possible roles of the central noradrenergic and NPY systems in the etiology of the hypercortisolemia of endogenous depression and anorexia nervosa are discussed. (*J. Clin. Invest.* 1994. 93:1439–1450.) Key words: hypothalamic-pituitary-adrenal axis • central facilitation • brain catecholaminergic pathways • brain neuropeptide Y pathways • psychiatric illness

Introduction

It is currently believed that the secretion of ACTH by the anterior pituitary is regulated in a unidirectional manner by the hypothalamus. This control is stimulatory in nature and is effected by a number of neuropeptides that are secreted into the hypophysial-portal circulation (1, 2). In the rat, corticotropin-releasing factor (CRF)[1] is the most potent ACTH secretagogue and the only neuropeptide known to augment pro-opiomelanocortin (POMC) biosynthesis (3, 4). The effect of CRF on ACTH release is potentiated by arginine vasopressin (AVP), oxytocin, angiotensin II, and the catecholamines norepinephrine and epinephrine (5, 6). However, it is now apparent that CRF is not the most potent ACTH secretagogue in all species since CRF and AVP are equipotent in their ability to release ACTH from bovine anterior pituitary cells (7), and in the ovine species, AVP is a more potent ACTH secretagogue than is CRF in vivo and in vitro (8–10). In addition, AVP also increases total ACTH accumulation in cultured ovine anterior pituitary cells, a finding that has been interpreted to reflect an effect of AVP on POMC biosynthesis (10).

The CRF and AVP neurons that project to the median eminence and secrete into the hypophysial-portal circulation are mainly located in the medial parvocellular subdivision of the paraventricular hypothalamus (PVHmp) (11). Recent immunohistochemical studies performed in the rat indicate that pro-AVP expressing and pro-AVP–deficient CRF perikarya are found in almost equal proportions in the PVH (12). Although comparative studies have yet to be performed, it is probable that similar findings may be obtained in other species. In the rat, the CRF_+/AVP_+ subpopulation is preferentially concentrated in the dorsolateral aspect of the PVHmp (12), and this area is densely innervated by dopamine-β-hydroxylase (DBH) and phenylethanolamine-*N*-methyltransferase (PNMT) nerve terminals (13). The DBH-immunoreactive (ir) axon terminals in the PVH are derived from noradrenergic cell bodies located in the nucleus of the tractus solitarius (NTS, A2 area), the ventrolateral medulla (A1 area), and the locus ceruleus (A6 area), whereas the PNMT-ir fibers originate from the C1, C2, and C3 brainstem adrenergic cell groups. The adrenergic cell groups are found rostral to the noradrenergic perikarya and the C1 group appears to be absent from the ovine brain (13, 14). There are direct synaptic contacts between these DBH- and PNMT-ir nerve fibers and the CRF-stained neurons in the PVH, suggesting that the ascending noradrenergic and adrenergic pathways are strategically placed to regulate CRF and AVP secretion and/or biosynthesis (15).

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1. *Abbreviations used in this paper:* AVP, arginine vasopressin; CRF, corticotropin-releasing factor; DBH, dopamine-β-hydroxylase; icv, intracerebroventricular; EPI, epinephrine; ir, immunoreactive; LC, locus ceruleus; NE, norepinephrine; NPY, neuropeptide Y; NTS, nucleus of the tractus solitarius; PNMT, phenylethanolamine-*N*-methyltransferase; POMC, pro-opiomelanocortin; PVHmp, paraventricular hypothalamus.

Neuropeptide Y (NPY) is a highly conserved 36-residue peptide that was isolated from porcine brain and subsequently found to be widely distributed in the mammalian central and peripheral nervous system (16). The concentrations of NPY in the mammalian brain are higher than those of any previously discovered neuropeptide and the hypothalamus contains particularly high concentrations of the peptide (17–19). Studies using immunohistochemistry and in situ hybridization histochemistry have determined the distribution of NPY-ir perikarya and fibers throughout the brain (17–22). The hypothalamic arcuate nucleus contains the highest concentration of NPY-ir perikarya of any brain region, and from this nucleus there arises a prominent projection that proceeds rostrally to innervate the PVH (19, 23). NPY is not colocalized with either norepinephrine (NE) or epinephrine (EPI) within the arcuate nucleus (23), but in the brainstem, NPY is extensively colocalized within the adrenergic neurons of the C1, C2, and C3 groups, while its correspondence within the noradrenergic cell groups is less complete (21). The NPY-ir axon terminals within the PVH are therefore derived from two sources, the arcuate nucleus and the brainstem, and of these two the arcuato-paraventricular projection is the more important. Furthermore, NPY-ir axon terminals make direct synaptic contacts with CRF-stained perikarya, raising the possibility that NPY may also regulate CRF and AVP secretion and/or biosynthesis (24).

A number of recent studies have demonstrated that CRF and AVP are present in the hypophysial-portal circulation of the rat and the sheep (25–30). We have recently demonstrated that CRF and AVP are secreted in a pulsatile fashion into the hypophysial-portal circulation of the conscious ewe and that insulin-induced hypoglycemia markedly increases AVP and, to a lesser extent, CRF concentrations in portal plasma (29). This marked increase in AVP release increases the AVP/CRF molar ratio and is consistent with the suggestion that insulin-induced hypoglycemia activates the CRF_+/AVP_+ perikarya and/or the magnocellular AVP neuronal system. A number of brain regions, including the NTS, the lateral hypothalamic area (LHA), and the ventromedial hypothalamus (VMH), contain neurons that respond to changes in glucose concentration by altering their firing rates (31–33). The PVH is not thought to contain glucose-responding neurons, and although hypoglycemia augments the in vitro secretion of CRF from rat hypothalamic fragments, such studies cannot reliably distinguish between a direct effect of glucose on PVH CRF-containing neurons and an indirect effect that is mediated by the VMH or some other hypothalamic region (34). Therefore, the effect of hypoglycemia on CRF and AVP release is likely to be indirect and secondary to a primary activation of one or more of the aforementioned glucose-sensitive regions. Since hypoglycemia increases the turnover of NE in the hypothalamus (35), we have suggested that the effect of hypoglycemia on neuronal firing in the NTS may somehow be translated into an increased synthesis of NE within the NTS itself or within the A1 and A6 areas, since all three regions are interconnected (13, 29). This conceptual framework would explain the increased hypothalamic turnover of NE during hypoglycemia, and would predict that NE might act on CRF-ir perikarya in the PVH to stimulate the release of CRF and/or AVP.

As a preliminary step towards verifying this prediction, we initially assessed the effect on the pituitary-adrenal axis of injecting either NE or EPI intracerebroventricularly in the conscious sheep (36). In those studies, we noted that NE and EPI caused both an acute and sustained increase in ACTH and cortisol secretion, and that NE appeared to be the more potent agonist in vivo. Since NE and EPI released only very modest amounts of ACTH from the anterior pituitary, we concluded that both catecholamines were acting mainly at suprahypophysial brain sites to increase CRF and AVP secretion. In the studies described below, we have directly measured the effects of intracerebroventricular (icv) NE on the secretion of CRF and AVP into the hypophysial-portal circulation as well as ACTH and cortisol into the systemic circulation of the conscious sheep. Since the distribution of NPY-stained varicosities in the parvocellular PVH encompasses the PNMT-ir and DBH-ir input (21), we have also assessed the effect of icv NPY on the activity of the entire hypothalamic-pituitary-adrenal axis. A preliminary account of these observations has appeared in abstract form (37).

Methods

Animals. Mature ovariectomized ewes were anesthetized (6 ml nembutal and 4 ml thiopentone intravenously followed by 3–5% halothane in 0_2) and bilateral stainless steel guide cannulae were inserted to access the lateral ventricles. The animals were allowed to recover and were housed in individual pens, allowed ad libitum access to food and water, and were handled repeatedly.

Several weeks later, these animals were anesthetized again and the pituitary fossa was approached via the transnasal, transsphenoidal route. The hypothalamo-hypophysial portal vessels were exposed, and two 12-gauge stainless steel guide needles were secured in place with one fixed 3 mm from the pituitary gland (38). The animals were again allowed to recover, and 3 d later an indwelling catheter was inserted into the external jugular vein and the patency and depth of the icv cannula were determined. The experiment was performed on the morning of the following day. A needle was passed through one of the transsphenoidal guide tubes to lesion some of the portal vessels coursing over the anterior aspect of the pituitary gland, and the other cannula was connected to a suction apparatus and was used to aspirate pituitary portal blood.

Experimental design. The experiments were commenced at 0900 h and were of 8-h duration. The baseline state was established by obtaining blood samples at 10-min intervals over 4 h, after which each animal received an icv injection. During the half-hour period that immediately followed the icv injection, peripheral blood samples were collected at 1, 2, 5, 10, 15, 20, and 30 min, and portal blood samples were collected at 10, 20, and 30 min. Thereafter, the 10-min sampling interval was resumed and continued for a further 3.5 h.

The dose of 50 μg NE that was chosen for these experiments was based on our previous studies of the hypothalamic-pituitary-adrenal axis in the conscious sheep. In experiments performed with sheep bearing only indwelling internal jugular venous cannulae, we have demonstrated that 10 μg icv NE causes acute 1.9- and 3.2-fold increases in mean plasma ACTH and cortisol levels over the 1-h period postinjection, and 1.6- and 2.3-fold increments in their concentrations over the 4-h postinjection period (36). In those studies, the basal plasma cortisol levels during the 4-h preinjection period were 10–20 nmol/liter. However, in our studies of CRF and AVP secretion in conscious sheep bearing both hypophysial-portal and internal jugular venous cannulae, we noticed that plasma cortisol levels at the onset of sampling were greatly elevated (100 nmol/liter) in some animals (29). We have attributed this finding to acute stress in these animals, and we therefore increased our doses of NE fivefold (to 50 μg) in the current studies in anticipation of its occurrence.

The dose of NPY used in these studies was chosen on the basis of unpublished experiments in the conscious sheep in which we observed

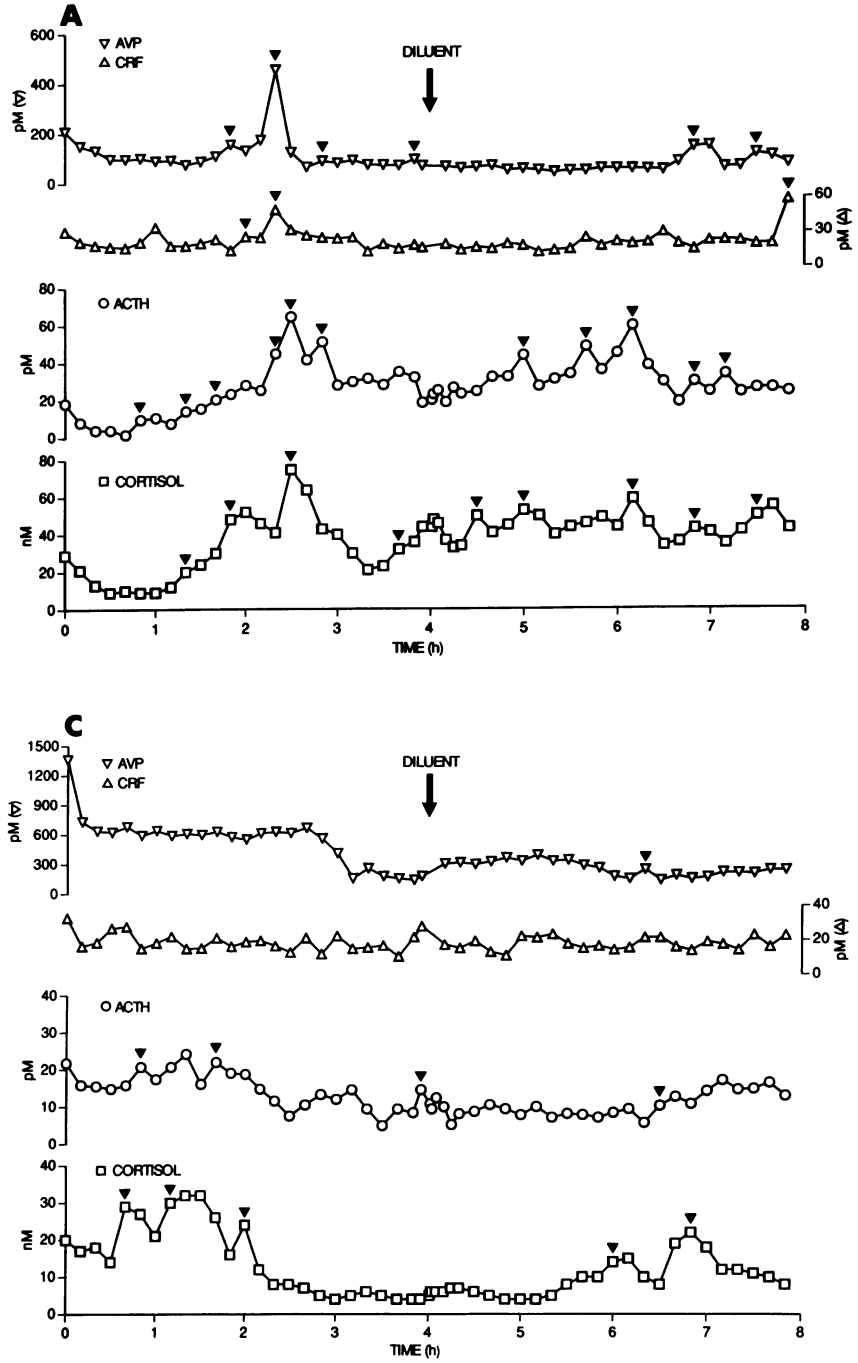

Figure 1. The effect of an icv injection of NaCl (diluent) on plasma CRF, AVP, ACTH, and cortisol levels in three ovariectomized ewes. In this and the subsequent two figures, (*top*) CRF and AVP values, (*middle*) ACTH values, and (*bottom*) cortisol values. The arrows depict the time of injection and the triangles depict significant hormone pulses.

that 50 μg icv NPY caused a very robust and prolonged increase in ACTH and cortisol secretion.

Three groups of animals (n = 3 per group) were studied: group 1 consisted of the control animals which received 40 μl icv sterile 0.9% physiological saline (NaCl); group 2 were given 50 μg icv NE in 40 μl NaCl, and group 3 received 50 μg icv NPY in 40 μl NaCl. At the end of the experimental day, the animals were killed, and the localization of the ventricular and portal cannulae were verified.

Ovine anterior pituitary cell culture. Ovine pituitary glands were obtained from a local abattoir and transported to Prince Henry's Institute of Medical Research at 4°C. The anterior lobe was separated from the intermediate and posterior lobes, minced, and then digested with 0.5% trypsin and 0.4% DNase (10). The dispersed anterior pituitary cells (viability, > 95%) were plated at a density of 3.3×10^5 cells/ml and cultured at 37°C in an environment of 5% CO_2 in air for 3 d before use. On the day of the experiment, the cells were exposed to the test substances for 4 h, after which the media were removed and stored at −20°C until use in the ACTH RIA.

Extraction and measurement of ir-CRF and ir-AVP in hypophysial-portal plasma. Hypophysial-portal blood was collected into tubes (at 4°C) containing trasylol (2,100 U/ml), *N*-ethylmaleimide (0.2 M), and disodium ethylenediamine-tetraacetate (50 mM). The samples were centrifuged immediately and the plasma stored at −20°C. Subsequently, each sample was loaded onto a separate Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) and the peptides were eluted with 3 ml 80% acetonitrile/0.1% trifluoroacetic acid. The eluates were dried and reconstituted in 1 ml buffer just before use in the RIAs. The recoveries of CRF and AVP ranged between 50 and 60% and were estimated by subjecting an aliquot of ovine portal plasma containing a known amount of unlabeled CRF or AVP to this procedure. All the values have been corrected accordingly.

The CRF RIA was performed with a specific antiserum (kindly provided by Dr. Robert Benoit, Montreal, Canada) raised against synthetic ovine $_{(o)}CRF_{1-41}$, ^{125}I-CRF, and synthetic oCRF as the standard. The sensitivity of the RIA was 2 pg/tube, and the inter- and intraassay coefficients of variation were 12 and 8%, respectively. The portal plasma extracts (400 μl) were measured in duplicate, and the results (pg/ml) were converted to pmol/liter by dividing by the molecular weight of $_{o}$CRF (4,671).

The AVP RIA was performed with a specific antiserum (R151) raised against synthetic $AVP_{(1-9)}$, ^{125}I-AVP, and synthetic $AVP_{(1-9)}$ as the competing standard. The sensitivity of the method was 2 pg/tube, and the inter- and intraassay coefficients of variation were 11 and 8%, respectively. The portal plasma samples (50–100-μl aliquots) were assayed in duplicate and the results (pg/ml) were converted to pmol/liter by dividing by the molecular weight of AVP (1,084).

Statistics. The RIA data were analyzed with a computer program that estimates the intraassay coefficient of variation across the entire standard curve and that also provides the standard deviation for each assay result (39). An increase in the plasma level of each substance was designated to be a significant pulse if it satisfied two criteria: the value had to exceed the previous point by at least 3 SD, and the value had to be followed by a nadir or a statistically nonsignificant change. We have used these criteria in previous studies of CRF, AVP, and POMC-peptide secretion, and they provide a similar identification of pulses to the method of cycle detection (40).

As a preliminary step, the effects of the icv injections on the plasma concentrations of CRF, AVP, ACTH, and cortisol were individually analyzed by the repeated measurements (nested) analysis of variance. The entire experimental period was divided into eight 1-h periods and the mean concentrations of CRF, AVP, ACTH, and cortisol in each 1-h period were calculated from the mean values of the three animals in each experimental group. The data contain both within-animal and between-animal sources of random variation, and this is reflected by two error terms in the analysis of variance. The effect of treatment was tested against the between-animal variance, and the effect of time and the interaction between treatment and time were tested against the within-animal variance.

Subsequently, the temporal effects of the icv injections were analyzed by a t test. The differences between consecutive 1-h periods within each treatment group were divided by their standard error, and a P value was calculated from the t distribution. The effects of the injections were divided into two components, namely an acute effect that was measured by hormonal changes occurring within the first 1–2 h postinjection, and a chronic effect that was assessed by changes produced 4 h postinjection. The comparisons were made with the 1-h period immediately preceding the icv injections.

The data obtained from the in vitro experiments were also analyzed by the one-way analysis of variance followed by Duncan's multiple range test. All the data have been expressed as the mean±SE.

Results

Effect of icv NaCl. During the entire 4-h baseline period, the typical ultradian secretion of CRF, AVP, ACTH, and cortisol was observed (Fig. 1, *A–C*), although, as expected, the secretory patterns were markedly heterogeneous (29). In all three animals, the AVP concentrations and pulse amplitudes exceeded those of CRF, and these findings are consistent with our previous observations (29). In two of the animals (B and C), plasma concentrations of AVP, ACTH, and cortisol declined with time, suggesting that the hypothalamic-pituitary-adrenal axis was activated in these ewes at the onset of sampling.

When a comparison was made with the 4-h period (1 h preinjection), mean plasma levels of CRF, AVP, ACTH, and cortisol during the 5-h period (1 h postinjection) were unchanged. In addition, the mean plasma concentrations of each of these substances during the 8-h period (4 h postinjection) did not differ significantly from those measured during the 4-h period (1 h preinjection). Taken together, these findings indicated that the icv injection of NaCl caused neither an acute nor a chronic activation of the hypothalamic-pituitary-adrenal axis (Table I).

Effect of 50 μg icv NE. An examination of the hormonal profiles in this group also revealed the unpredictable basal hormone secretion, but also documented that the CRF, AVP, ACTH, and cortisol responses to icv NE were also heterogeneous (Fig. 2, *A–C*). At the onset of the experiment, portal plasma levels of CRF and AVP in animal A were markedly

Table I. The Effect of an icv Injection of NaCl (Diluent) on Mean Plasma Concentrations of CRF and AVP in the Hypophysial-Portal Circulation and ACTH and Cortisol in the Systemic Circulation of Three Ewes

Period of observation	Time	CRF	AVP	ACTH	Cortisol
	h	*pM*	*pM*	*pM*	*nM*
Preinjection	1	34±11*	341±207*	21±9*	43±25*
	2	26±7	280±159	19±1	36±7
	3	27±5	296±146	24±10	35±14
	4	19±0.4	109±35	20±6	27±12
Postinjection	5	18±1	149±88	18±5	36±16
	6	19±0.3	149±76	21±9	37±15
	7	21±1	101±39	20±7	37±11
	8	24±3	113±57	21±4	37±14

* Each value represents the mean±SE of the three animals during each 1-h time interval.

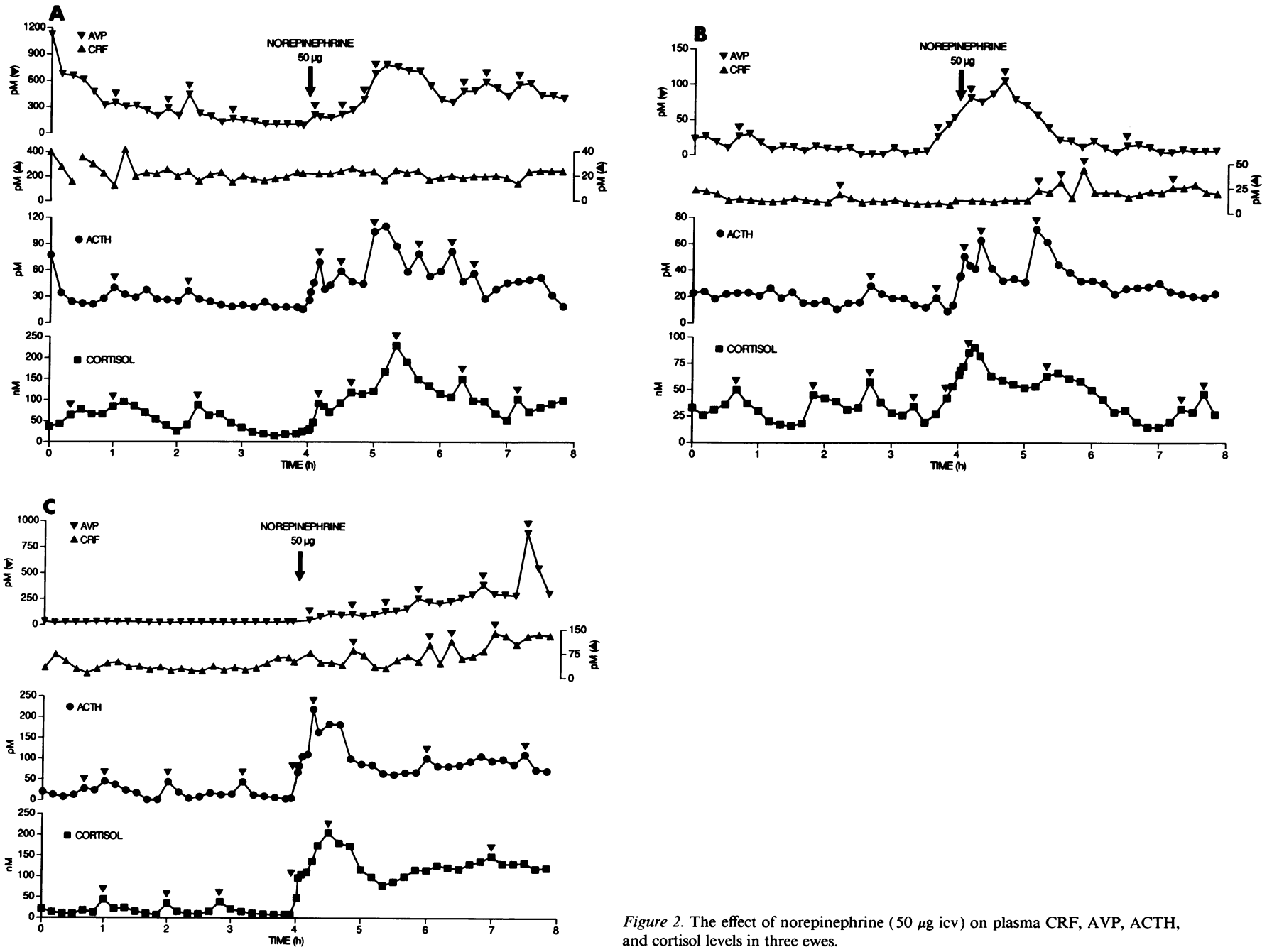

Figure 2. The effect of norepinephrine (50 μg icv) on plasma CRF, AVP, ACTH, and cortisol levels in three ewes.

increased and caused plasma cortisol levels to rise up to 100 nmol/liter. Plasma AVP, ACTH, and cortisol levels declined gradually throughout the basal period, suggesting that the hypothalamic-pituitary-adrenal axis was significantly activated in this animal at the outset. When compared with animal A, CRF and AVP concentrations in animals B and C were far lower, their secretory profiles were remarkably quiescent, and plasma cortisol levels did not exceed 50 nmol/liter.

A close inspection of these three animals' hormonal profiles revealed that significant pulsatile ACTH and cortisol secretion had occurred at 1 min after the icv injection. Although it was not technically possible to document increased hypothalamic ACTH-releasing factor secretion at 1 min postinjection, this rapid activation of the pituitary-adrenal axis was undoubtedly due to an increased pulsatile secretion of AVP and, to a lesser extent, of CRF. During the 1-h period that followed the icv injection of NE, mean ACTH and cortisol concentrations were significantly increased (Table II: ACTH, 74±28 vs. 15±2 pmol/liter, $P = 0.0001$; cortisol, 94±20 vs. 21±7 nmol/liter, $P = 0.0004$). Mean plasma CRF and AVP concentrations were increased at this time, but the rise in AVP levels only reached significance at the 6-h period (280±186 vs. 54±27 pmol/liter, $P = 0.0006$).

NE also caused a prolonged increase in hypothalamic-pituitary-adrenal axis activity, since mean plasma levels of CRF, AVP, ACTH, and cortisol were all increased at 4 h postinjection (Table II: CRF, 58±34 vs. 27±11 pmol/liter, $P = 0.04$; AVP, 310±153 vs. 54±27 pmol/liter, $P = 0.002$; ACTH, 49±20 vs. 15±2 pmol/liter, $P = 0.01$; cortisol, 82±28 vs. 21±7 nmol/liter, $P = 0.002$).

Effect of 50 μg icv neuropeptide Y. Plasma CRF, AVP, ACTH, and cortisol levels were elevated in all three animals at the onset of this experiment, indicating that the hypothalamic-pituitary-adrenal axis was significantly activated in this entire experimental group (Fig. 3, *A–C*). However, a steady decline in the levels of each substance was noted over the ensuing 1–2 h, suggesting that each ewe had adapted to the experimental conditions before the timing of the icv injection.

A close inspection of the three animals' hormonal profiles revealed that significant pulsatile secretion of ACTH and cortisol occurred 1 min after the icv injection. Although we could not document increased CRF or AVP secretion at 1 min postinjection, this rapid effect of NPY on the pituitary-adrenal axis was most likely due to increased secretion of AVP and CRF. During the 1-h period after the injection of NPY, mean AVP, ACTH, and cortisol concentrations were significantly increased (Table III: AVP, 181±99 vs. 43±18 pmol/liter, $P = 0.08$; ACTH, 134±23 vs. 26±3 pmol/liter, $P = 0.0001$; cortisol, 69±21 vs. 17±5 nmol/liter, $P = 0.008$). Although mean plasma CRF concentrations were increased (63±5 vs. 45±10 pmol/liter), the heterogeneity of the plasma CRF responses to icv NPY precluded this rise from attaining statistical significance ($P = 0.22$).

NPY also caused sustained ACTH hypersecretion since mean plasma ACTH levels were significantly increased at 4 h postinjection (Table III: ACTH, 67±13 vs. 26±3 pmol/liter, $P = 0.004$). At this time, mean AVP and cortisol levels were both increased 1.8-fold (AVP, 80±40 vs. 43±18 pmol/liter; cortisol, 31±4 vs. 17±5 pmol/liter), although the heterogeneity of the responses precluded these 4-h values from attaining statistical significance.

Effect of neuropeptide Y on the release of ACTH from cultured ovine anterior pituitary cells. To determine the predominant site of action of NPY when administered in vivo, we examined the effects of NPY on the basal, CRF-, and AVP-induced release of ACTH from cultured ovine anterior pituitary cells.

When added alone, NPY (10^{-11}–10^{-6} M, 4-h) did not cause any change in the secretion of ACTH. In addition, the amount of ACTH released by CRF (10^{-11}–10^{-7} M) or AVP (10^{-11}–10^{-6} M) was unaffected by the presence of 10^{-7} M NPY.

Table II. The Effect of an icv Injection of 50 μg NE on Mean Plasma Concentrations of CRF and AVP in the Hypophysial-Portal Circulation and ACTH and Cortisol in the Systemic Circulation of Three Ewes

Period of observation	Time	CRF	AVP	ACTH	Cortisol
	h	*pM*	*pM*	*pM*	*nM*
Preinjection	1	64±36*	217±191*	26±5*	39±13*
	2	25±6	98±79	23±3	35±13
	3	22±4	84±67	18±3	37±11
	4	27±11	54±27	15±2	21±7
Postinjection	5	34±15	157±72	74±28‡	94±20§
	6	35±11	280±186‖	65±9	107±31
	7	42±22	253±134	55±18	83±31
	8	58±34¶	310±153**	49±20‡‡	82±28**

* Each animal value represents the mean±SE of the three animals taken during each 1-h time interval. 5-h period (1 h after icv injection) vs. the 4-h period (1 h preinjection): ‡ $P = 0.0001$; § $P = 0.0004$. 6-h period (2 h after icv injection) vs. the 4-h period (1 h preinjection): ‖ $P = 0.0006$. 8-h period (4 h after icv injection) vs. the 4-h period (1 h preinjection): ¶ $P = 0.04$; ** $P = 0.002$; ‡‡ $P = 0.01$.

Discussion

These studies provide a direct demonstration that the icv injection of NE activates the hypothalamic-pituitary adrenal axis in the conscious sheep. Although the secretion of both CRF and AVP was increased, the rise in AVP was consistently greater than that of CRF and the AVP/CRF molar ratio was consequently increased. We have observed a similar alteration in the AVP/CRF molar ratio during insulin-induced hypoglycemia and, to a lesser extent, after the onset of an audiovisual emotional stress (29). The most likely site of action of the injected NE is the PVH, which lies adjacent to the wall of the third ventricle. In the rat, the PVH contains both CRF_+/AVP_+ and CRF_+/AVP_- perikarya in approximately equal proportion (12), and the medial parvocellular subdivision (PVHmp) contains those CRF and AVP perikarya that project to the external zone of the median eminence (11, 41). Moreover, the dorsolateral aspect of the PVHmp contains a preferential concentration of CRF_+/AVP_+ perikarya in direct synaptic contact with DBH-stained nerve fibers (15). Although comparable immunohistochemical studies have yet to be performed in the sheep, it is possible that a similar cytoarchitectonic organization might be found. Although the icv NE increased CRF and AVP concentrations in portal plasma and increased the AVP/CRF molar ratio, the increases in CRF and AVP levels were not temporally coincident. These findings suggest that in addition to activating parvocellular CRF_+/AVP_+ and/or CRF_+/AVP_-

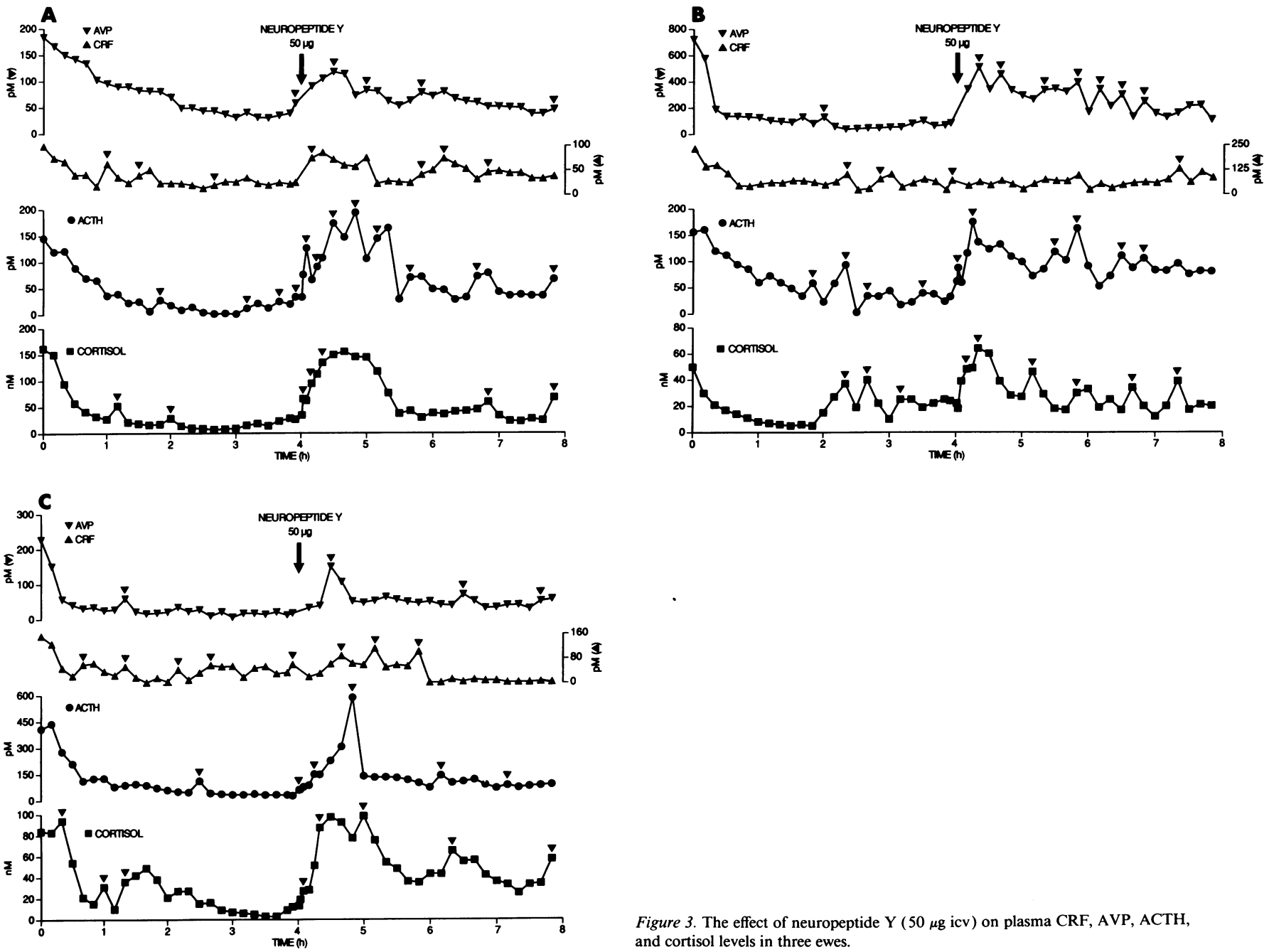

Figure 3. The effect of neuropeptide Y (50 µg icv) on plasma CRF, AVP, ACTH, and cortisol levels in three ewes.

Table III. The Effect of an icv Injection of 50 μg Neuropeptide Y on Mean Plasma Concentrations of CRF and AVP in the Hypophysial-Portal Circulation and ACTH and Cortisol in the Systemic Circulation of Three Ewes

Period of observation	Time	CRF	AVP	ACTH	Cortisol
	h	*pM*	*pM*	*pM*	*nM*
Preinjection	1	86±17*	170±62*	149±47*	52±17*
	2	43±13	71±23	50±16	22±8
	3	49±14	37±8	34±14	17±5
	4	45±10	43±18	26±3	17±5
Postinjection	5	63±5	181±99‡	134±23§	69±21‖
	6	57±11	140±81	101±7	45±8
	7	40±15	110±59	78±15	38±9
	8	47±27	80±40¶	67±13**	31±4

* Each value represents the mean±SE of the three animals taken during each 1-h time interval. 5-h period (1 h after icv injection) vs. the 4-h period (1 h preinjection): ‡ $P = 0.08$; § $P = 0.0001$; ‖ $P = 0.008$. 8-h period (4 h after icv injection) vs. the 4-h period (1 h preinjection): ¶ $P = 0.002$; ** $P = 0.004$.

neurons, the injected NE may have stimulated magnocellular AVP cell groups within the supraoptic and paraventricular nuclei. The axons derived from these perikarya run in the internal zone of the median eminence, make multiple contacts with the pericapillary space in this region, and release AVP-containing secretory granules (42–44). Furthermore, studies in the rat also suggest that most of the AVP in the hypophysial-portal circulation is derived from magnocellular sources since portal AVP concentrations are unaffected by bilateral PVH lesions or neurolobectomy (45, 46).

It is also possible that the injected NE indirectly stimulated the tuberoinfundibular CRF and AVP neurons by initially activating those paraventricular CRF neurons that form part of the autonomic nervous system. These perikarya send descending projections to the brainstem and some of these innervate the locus ceruleus (LC, A6 area, 11). The observation that the direct injection of CRF into the LC increases its rate of neuronal firing suggests that LC activity could be stimulated by endogenous CRF pathways (47). Moreover, the finding that increased neuronal firing in the LC is associated with increased hypothalamic concentrations of the NE metabolite 3,4-dihydroxyphenylglycol suggests that increases in LC activity cause an increased NE turnover in the nucleus that may then be relayed to the hypothalamus (48). Since the hypothalamus is only sparsely innervated by ascending noradrenergic projections from the LC, it seems unlikely that an increase in hypothalamic NE turnover could be simply due to increased noradrenergic activity occurring solely within the LC. As previously noted, however, the LC is connected to the NTS and ventrolateral medulla, suggesting the possibility that an increase in LC activity could be transmitted to these areas. Since the NTS, ventrolateral medulla, and LC all provide ascending noradrenergic projections to the hypothalamus via the medial forebrain bundle, an activation of descending autonomic CRF projections by icv NE could increase NE turnover in these three brainstem areas, which could then be relayed by the medial forebrain bundle to increase NE release in the hypothalamus. The endogenously released NE could in turn act upon parvocellular CRF_+/AVP_+ and CRF_+/AVP_- neurons and the magnocellular AVP neuronal system to increase CRF and AVP secretion into the hypophysial-portal circulation. This neuroanatomical circuitry could provide a link between the autonomic nervous system and hypothalamic neuropeptide secretion.

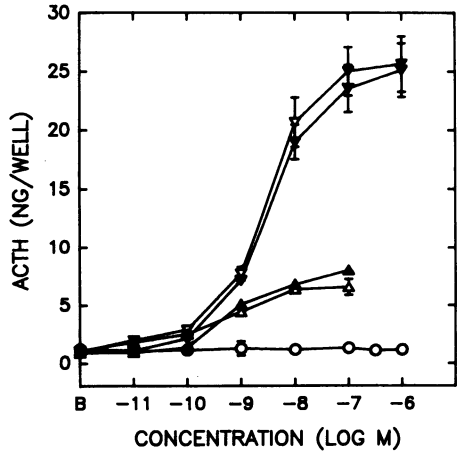

Figure 4. The concentration-dependent effects of neuropeptide Y on the release of ACTH (○) from dispersed ovine anterior pituitary cells. The concentration-dependent effects of CRF (△) and AVP (▽) on ACTH release and the effect of 10^{-7} M NPY added in combination with CRF (▲) or AVP (▼) are also shown.

To our knowledge, this study is the first to demonstrate that icv NE simultaneously increases the secretion of both ir-CRF and ir-AVP into the hypophysial-portal circulation. The finding that NE is capable of stimulating CRF secretion is entirely consistent with a number of recent in vivo and in vitro studies performed in the rat. The studies of Plotsky (49) have shown that 0.1–5 nmol icv NE increases the in vivo release of CRF and that this effect is attenuated by the α_1-receptor antagonist coryanthine. Conversely, Szafarczyk et al. (50) have demonstrated that catecholaminergic denervation of either the PVH or the whole hypothalamus significantly reduces the level of ir-CRF in portal plasma, and have suggested that the central catecholaminergic innervation stimulates CRF release by acting both at the level of the PVH and the external zone of the median eminence. In addition, a number of in vitro studies indicate that NE increases CRF secretion from hypothalamic explants or dispersed hypothalamic cells. However, controversy exists regarding the type of adrenergic receptor that mediates these responses, since Calogero et al. (51) have suggested that the effect is mediated by both α_1 and α_2 adrenergic receptors, whereas Tsagarakis et al. (52) and Widmaier et al. (53) have proposed that the action is mediated through the β-adrenergic receptor.

The glucocorticoids are thought to exert rapid, intermediate, and slow negative feedback effects over ACTH release and biosynthesis, and these time domains have been established by measuring the pituitary-adrenal response to a stressful stimulus applied at various periods after the administration of exogenous glucocorticoid (54). For example, fast feedback operates within seconds to minutes of glucocorticoid administration at a time when plasma concentrations of the hormone are rising, and appears not to involve an effect on CRF or POMC biosynthesis. The intermediate feedback appears after short durations of exposure to glucocorticoid, is maximally evident between 2 and 4 h after steroid administration, and may

involve an inhibition of CRF biosynthesis. Slow feedback only appears after prolonged exposure (> 24 h) to medium or high concentrations of glucocorticoids and causes a decrease in POMC biosynthesis. In this study, glucocorticoids were not administered at any time before the icv injection since the study was not designed to investigate the temporal characteristics of glucocorticoid-negative feedback on the ovine pituitary or brain. However, it was noted that two of the animals (Fig. 2, *A* and *B*) displayed marked hypercortisolemia at the onset of the experiment, suggesting that they were acutely stressed at this time. In spite of the hypercortisolemia, these animals also demonstrated an exuberant hypothalamic-pituitary-adrenal response to the icv NE, although the catecholamine was injected when the intermediate glucocorticoid feedback should have been maximally operative. These findings are in accord with studies in the rat that demonstrate that elevations in endogenous plasma corticosterone consequent upon exposure to a stressful stimulus do not abrogate the pituitary-adrenal response to a second stress. Akana and Dallman (55) have termed this phenomenon "facilitation" and it is likely that multiple central mechanisms underly its occurrence.

The protocol used in this study was designed to pharmacologically mimic a metabolic stimulus such as insulin-induced hypoglycemia, which is known to increase hypothalamic NE turnover (35). We noted that the icv NE caused both an acute and sustained activation of the hypothalamic-pituitary-adrenal axis, indicating that hypersecretion of AVP and CRF may occur in the presence of greatly elevated levels of cortisol. Although the subcellular mechanisms that underly the sustained hypothalamic-pituitary-adrenal activation cannot be elucidated by an in vivo approach, we speculate that the following mechanisms may be involved. First, the icv NE might activate a CRF neuronal subpopulation that is relatively insensitive to glucocorticoid-negative feedback, a possibility that is supported by the finding of a CRF neuronal subpopulation in the PVHmp that does not appear to express the glucocorticoid receptor (56). Second, the activation of hypothalamic adrenergic receptors by icv NE might stimulate CRF and AVP biosynthesis to such an extent that the glucocorticoid negative feedback effect on CRF and AVP gene expression is attenuated or abolished. The suggestion that the central noradrenergic pathways might stimulate CRF biosynthesis in vivo is supported by the observations that insulin-induced hypoglycemia increases both the turnover of NE and the level of CRF mRNA expression in the hypothalamus (35, 57). NE also stimulates the in vitro release and biosynthesis of CRF in fetal rat hypothalamic cells, and this effect involves the activation of both the A and C protein kinases and is mimicked by the phorbol ester TPA (58, 59). TPA activates protein kinase C and increases the expression of the two major constituents of the AP-1 transcription factor complex, namely the protooncogene products Fos and Jun (60). In this regard, it is pertinent that the overexpression of either Fos or Jun may disrupt the binding of the glucocorticoid receptor to the glucocorticoid regulatory element in a number of systems and repress the glucocorticoid inhibitory effect on gene expression (61). If a similar interaction also occurred in PVH CRF_+/AVP_+ neurons in response to NE, it might partly explain the finding of persistent CRF and AVP hypersecretion occurring in the face of sustained hypercortisolemia.

These observations may be of clinical relevance in that subgroup of patients with endogenous depression who display hypercortisolemia. These patients show a marked increase in ACTH pulse frequency, although the ACTH pulse amplitude and mean ACTH concentrations are normal (62, 63). The hypercortisolemia is associated with cortisol pulses that are of increased amplitude and duration (63), and these changes may be partly due to an increased adrenocortical sensitivity to ACTH (64). However, the finding that plasma cortisol levels fail to suppress into the subnormal range on the morning after the administration of dexamethasone (1 mg, 2300 h) implies a coexistent central dysregulation of the hypothalamic-pituitary-adrenal axis (65). The possibility that CRF hypersecretion is a component of this central dysregulation is supported by the recent study of Ur et al. (66), which has demonstrated an exaggerated ACTH response to metyrapone in depressed "cortisol nonsuppressor" patients. When compared with depressed "cortisol suppressor" patients and control subjects, depressed cortisol nonsuppressor patients also display increased CSF levels of the NE metabolite MHPG and an increased urinary excretion of NE and normetanephrine (67). Although those patients with endogenous depression demonstrate an increased rate of entry of NE into plasma ("increased NE spillover rate"), they do not display significant symptoms and signs of increased sympathetic nervous system activity. These findings, together with the recent demonstration of unidirectional spillover of NE from the brain into the systemic circulation (68), suggest that the apparent increase in NE spillover in endogenous depression may be due to enhanced central noradrenergic activity rather than to an increased release of NE from sympathetic nerve endings. From the findings in this study, we suggest that central noradrenergic activation may be an early event in depressed cortisol nonsuppressor patients and contribute to the hypothalamic-pituitary-adrenal dysregulation by causing hypersecretion of CRF and AVP.

The findings reported here may be of even greater human relevance when one considers that the icv NE injections mimicked even more closely the pituitary-adrenal secretory pattern that occurs during critical illness. During hospitalization in intensive care units, critically ill patients display chronic elevations of plasma cortisol levels that are positively correlated with the severity of the illness and are associated with elevated, or normal, levels of ACTH (69). The resetting of the hypothalamic-pituitary-adrenal axis in these patients may also be partly due to an attenuation of the normal glucocorticoid feedback, since morning plasma ACTH and cortisol concentrations are only minimally reduced by dexamethasone (3 mg, 2300 h). Although the pituitary-adrenal response to dexamethasone during critical illness is similar to that observed in patients with endogenous depression, the maximum ACTH response to CRF in the two clinical states differs, since the response is blunted in depression and augmented in critical illness (62, 69).

To our knowledge, this study is also the first to demonstrate that icv NPY increases the secretion of both CRF and AVP into the hypophysial-portal circulation of any animal species. NPY appeared to exert its effect on the hypothalamic-pituitary-adrenal axis by acting at one or more suprahypophysial brain sites since it did not stimulate ACTH secretion from ovine anterior pituitary cells. The action of NPY therefore contrasts with NE and EPI, which stimulate the hypothalamic-pituitary-adrenal axis by acting both at, and above, the pituitary level (36). The pattern of CRF and AVP secretion produced by NPY was identical to that produced by icv NE and insulin-induced hypoglycemia, since the rise in AVP was greater than that of CRF and the AVP/CRF molar ratio was consequently

increased. However, as previously noted for NE, the increases in CRF and AVP levels were not temporally coincident, suggesting that NPY may have primarily activated the magnocellular AVP system in addition to the CRF_+/AVP_+ and the CRF_+/AVP_- populations in the PVH. As previously suggested for NE, NPY may have also stimulated paraventricular autonomic CRF neurons, thereby increasing NE turnover in the A1, A2, and A6 areas, and secondarily increasing hypothalamic NE release and CRF and AVP secretion into the hypophysial-portal circulation. The findings reported here are consistent with recent studies performed in the rat and the dog, which also indicate that the central injection of NPY activates the pituitary-adrenal axis (70, 71).

The acute effect of 50 μg icv NPY on the hypothalamic-pituitary-adrenal axis was similar to, but somewhat less marked than, that of 50 μg icv NE. Although the sustained effect of NPY was even less pronounced than its acute effect, the 50-μg dose of NPY did cause a persistent increase in ACTH secretion. This effect of NPY must be ascribed to its ability to increase the hypothalamic release of AVP and, to a lesser extent, of CRF, since NPY did not exert a direct effect on ACTH secretion at the anterior pituitary level. It is quite possible that a higher dose of the peptide (≥ 100 μg icv) would reproduce the chronic effects of 50 μg icv NE on the hypothalamic-pituitary-adrenal axis, and further dose-response studies are required to confirm this postulate. The recent in vivo studies of Suda et al. (72) have demonstrated that NPY increases rat hypothalamic CRF mRNA levels at 2 h after its icv injection, thereby raising the possibility that NPY might also produce a similar effect in the ovine brain, although this remains to be formally demonstrated. However, the present findings are consistent with the suggestion that activation of the central NPY receptors may reset the activity of the hypothalamic-pituitary-adrenal axis. Although this study has not elucidated the subcellular mechanisms that underly this phenomenon, the possibilities outlined above to explain the identical effect of icv NE may apply equally to NPY and are amenable to further investigation.

The finding that NPY may chronically reset hypothalamic-pituitary-adrenal axis activity may assume clinical relevance when one considers both the role of NPY in the regulation of ingestive behavior and the state of the central NPY system and the hypothalamic-pituitary-adrenal axis in anorexia nervosa. NPY is one of the most potent endogenous stimulants of appetite in the brain because icv or direct paraventricular injections of NPY cause a marked stimulation of feeding behavior that can override mechanisms of satiety and body weight control (73–75). Moreover, short-term starvation in the rodent increases endogenous NPY secretion into the PVH, which is reversed by refeeding, suggesting that food deprivation may selectively activate the ARC-PVH NPY system (75). Although surprising, NPY levels are also increased in the CSF of underweight patients with anorexia nervosa (76). However, the coexistence of a disorder characterized by anorexia with increased CSF levels of a neuropeptide with potent orexigenic properties may perhaps be reconciled by the observation that a proportion of these patients are hungry but their hunger is overridden by a dysphoria that accompanies food ingestion. Anorexia nervosa is also characterized by slightly raised levels of ACTH and significant hypercortisolemia (77), and both the elevated cortisol production rates and CSF NPY concentrations decline to normal with the resumption of normal body weight (76, 78). Taken together with the findings of this study, we suggest that ARC-PVH NPY activity may be increased in anorexia nervosa and partly account for the elevated CSF NPY levels and the hypothalamic-pituitary-adrenal dysregulation that characterizes this disorder.

In summary, these studies have provided evidence that stimulation of either the central noradrenergic or NPY pathways causes both an acute and sustained activation of the hypothalamic-pituitary-adrenal axis in the conscious sheep. We suggest that these brain pathways may be of fundamental importance in activating and resetting the hypothalamic-pituitary-adrenal axis, which occurs in a variety of stressful stimuli. The hypotheses that increased central noradrenergic or NPY activity may partly account for the hypercortisolemia that occurs in some patients with endogenous depression or anorexia nervosa, respectively, may be amenable to further investigation with current brain imaging techniques.

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