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Evidence for a role of endogenous corticotropin-releasing factor in cold, ether, immobilization, and traumatic stress

(ACTH secretion/immunoneutralization/neural mediation)

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ABSTRACT **The role of corticotropin-releasing factor (CRF) in four model stresses (cold, ether, immobilization, and trauma) was examined in the guinea pig by using passive immunoneutralization with anti-CRF antiserum. Plasma corticotropin levels were measured at various times after exposure to stress, and groups treated with CRF antiserum were compared with those treated with normal rabbit serum. Of the four stresses tested, ether had the most pronounced effect on corticotropin secretion. Treatment with anti-CRF inhibited most of the ether-induced corticotropin secretory response, the difference between the normal serum- and the anti-CRF antiserum-treated groups being significant at 5 and 10 min ($P < 0.01$). Corticotropin responses to cold stress in the two groups differed at the 0.05 level of significance at 10 and 20 min. After administration of trauma (leg fracture), a statistically significant difference ($P < 0.01$) between the two groups also was evident, albeit only at 20 min. During immobilization, corticotropin levels differed significantly from control only in the normal serum-treated group but not in the anti-CRF-treated group. These findings show that CRF antiserum was effective in reducing corticotropin levels, indicating that CRF has an important role in mediating corticotropin response to stress. The fact that neutralization was incomplete might be due to an inability of the antiserum to sufficiently neutralize the endogenous CRF or, more likely, reflects the contribution of additional mediators, notably catecholamines and vasopressin, of corticotropin release upon stress.**

Corticotropin-releasing factor (CRF) has long been postulated to have a physiological role in modulating corticotropin (adrenocorticotropic hormone, ACTH) and β-endorphin secretion (1–3). Recent elegant studies in the rat (4) strongly imply that CRF and/or an endogenous peptide immunologically related to CRF has a physiologic role in the maintenance of elevated levels of circulating corticotropin in adrenalectomized rats and in the activation of the pituitary–adrenal axis due to ether stress. However, diverse stresses differ considerably in their pathophysiology, sequelae, and underlying neural control of corticotropin secretion (5–20). Therefore, these studies were undertaken utilizing a similar *in vivo* paradigm in the guinea pig to ascertain whether CRF performs an analogous role in other forms of stress. The results of the passive immunoneutralization approach (4, 21), which in this instance entails intravenous administration of a potent antibody against ovine CRF (oCRF) to the guinea pig before exposure to cold (6, 15, 19), trauma (8, 9), immobilization (14, 15, 17), or ether stress (5, 10, 11, 18), suggest that an endogenous CRF-like peptide does play a physiological role in the regulation of corticotropin in these model stresses.

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METHODS

A polyclonal antibody (hereafter referred to as anti-CRF) from rabbit 471 was used for the *in vivo* neutralization studies. It was prepared by the same methods used previously to raise antiserum for a highly sensitive radioimmunoassay for CRF (22, 23). As confirmed by logit-log transformations of the data and statistical comparisons of the resultant regression lines, this antiserum to oCRF displayed parallelism to extracts of guinea pig hypothalamus and extra-hypothalamic brain tissue (unpublished data). It was found that, after an overnight incubation period, anti-CRF at dilutions of 1:3000 and 1:300 bound >81% and >90%, respectively, of [[^{131}I]iodotyrosine]oCRF. The K_a was 1.24×10^{-12} M. In the high concentrations used for neutralization, the antiserum remained specific for oCRF and human/rat CRF (hrCRF) and displayed no crossreactivity to the various neuropeptides and anterior pituitary hormones tested, aside from the structurally related sauvagine with which the crossreactivity was <0.5%.

Synthetic CRF reference was dissolved in isotonic saline solution containing ascorbic acid and bovine serum albumin, as described by Orth *et al.* (24). Hartley male guinea pigs (Charles River Breeding Laboratories) weighing 350–450 g were maintained at a constant ambient temperature of 22 ± 1°C on an 11-hr light/13-hr dark cycle and given food and water ad lib. All guinea pigs in this study had been implanted with chronic indwelling intra-arterial catheters. For implantation, animals were anesthetized with sodium pentobarbital (25 mg/kg). A Silastic cannula (medical-grade tubing, 0.051 cm i.d., 0.094 cm o.d.; Dow Corning, Midland, MI) was placed through the right atrium, and the catheter then was threaded through a subcutaneous tunnel to the retroauricular region. When not in use for serial blood sampling, the cannula was filled with a heparin/saline solution. After surgery, rats were returned to individual Plexiglas one-way observation cages in a quiet environment for 2 days of recovery and handling.

All experiments were carried out in the morning, 48 hr after the implantation operations. Half an hour before the oCRF or the stress to be studied was administered, 0.5 ml of anti-CRF or normal rabbit serum (NRS) was injected through the catheter. Blood samples (0.2 ml) were taken in heparinized syringes at various times (see *Results*). To minimize decrements in blood volume over the short-term experimental period, isotonic saline solution (0.2 ml) was injected after each blood sampling. Animals were subjected to one of four stresses: ether, cold, unilateral leg fracture, or immobilization. Ether stress was caused by placing the animals for 1 min in a jar containing an ether-dampened paper at its bottom. Animals were exposed to cold stress by placing

Abbreviations: CRF, corticotropin-releasing factor; oCRF, ovine CRF; NRS, normal rabbit serum.

them in a plastic tank (18 × 30 × 15 cm) partially filled with water at 4°C for 3 min. For traumatic stress, the right hind tibia and fibula of each animal was broken manually and instantaneously. Animals were immobilized in a prone position with adhesive tape fastening the neck, trunk, and extremities onto a metal board.

Corticotropin Assay. Plasma corticotropin was determined by RIA with an antiserum (purchased from IgG Corporation, Nashville, TN) directed against the steroidogenic amino-terminal (residues 1–18) sequence of the corticotropin molecule (25). The reference preparation was synthetic human corticotropin[1–39] (Peninsula Laboratories, San Carlos, CA), and the tracer was ^{131}I-labeled human corticotropin. Aliquots (20 μl) of unextracted plasma were utilized for the RIA. To correct for any nonspecific effects on the assay of guinea pig plasma, aliquots of unextracted plasma, stripped of corticotropin with dextran-coated charcoal, were added to the standards used in the construction of the standard curve. Bound and free fractions were separated with talc. When the RIA was performed in this fashion, the sensitivity was 0.5 pg/tube and the intra- and interassay coefficients of variation were 7.7% and 11.9%, respectively. The effects of stress on plasma corticotropin levels were analyzed statistically by one-way analysis of variance. In addition, analyses comparing those groups treated with anti-CRF with those treated with NRS were carried out with the Student *t* test.

RESULTS

Administration of Synthetic oCRF. Increments in plasma corticotropin in response to the administration of increasing concentrations of oCRF are shown in Fig. 1. Ten guinea pigs were used for each dose of oCRF. Mean basal plasma corticotropin levels in the guinea pigs were 58.8 ± 9.5 pg/ml (mean ± standard error). Administration of oCRF in doses ≥0.1 μg induced a significant increase in plasma corticotropin by 5 min after injection, the earliest time point examined. An apparent dose relationship for plasma corticotropin response was obtained between 0.1 μg and 2 μg of oCRF. As there was no significant difference between the corticotropin responses following the 2- and 10-μg injections of CRF, the 2-μg dose was chosen for a subsequent pilot neutralization experiment (Fig. 2). After administration of this dose of CRF combined with NRS, plasma corticotropin levels increased dramatically. However, this increase was significantly inhibited throughout the sampling period ($P < 0.025$ at 5 and 10 min; $P < 0.01$ at 20 min) when CRF administration was accompanied by treatment with anti-CRF. Mean plasma corticotropin concentrations after administration of either NRS (68.9 ± 14.9 pg/ml) or antiserum alone (70.2 ± 9.1 pg/ml) did not differ significantly from control levels.

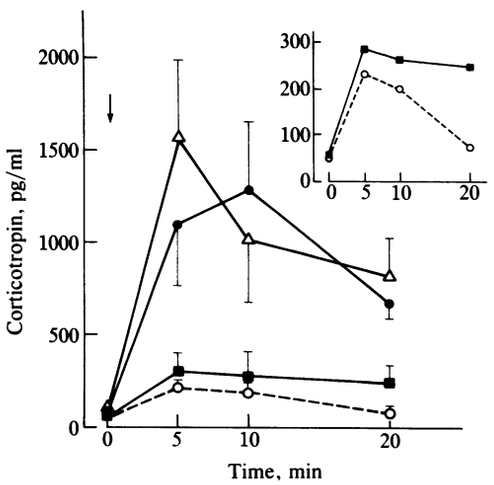

FIG. 1. Acute increments in plasma corticotropin in response to the administration (arrow) of 0.1 (○), 0.5 (■), 2 (●), or 10 μg (△) of oCRF. Ten guinea pigs were used for each dose of oCRF. *Inset*: Expanded vertical scale to show more clearly the plasma corticotropin response to 0.1 μg (○) and 0.5 μg (■) of CRF.

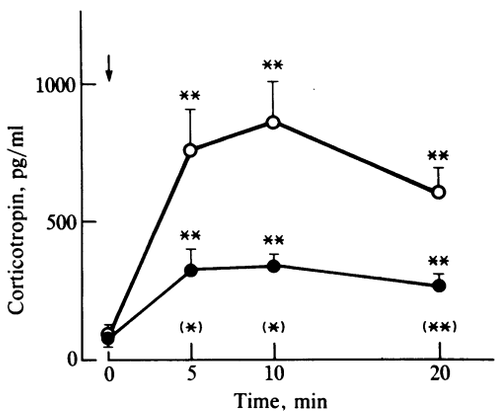

FIG. 2. Increments in plasma corticotropin in response to administration (arrow) of 2 μg of oCRF with NRS (○) or with anti-CRF (●). Eight guinea pigs were used for each treatment. In this and subsequent figures, significance of differences from control are indicated by symbols above the points; symbols in parentheses indicate significance of differences between corticotropin concentrations in NRS- and anti-CRF-treated animals at the various times. *, $P < 0.05$; **, $P < 0.01$.

Ether Stress. Plasma corticotropin concentrations in guinea pigs after a 1-min exposure to ether vapor are illustrated in Fig. 3. Among the various stresses to which the animals were subjected, ether had the most marked effect on corticotropin secretion. Peak values of plasma corticotropin were obtained within 5 min after the ether stress, the earliest point studied. Thereafter, the corticotropin concentration decreased gradually in the NRS-treated group but did not return to normal by 20 min, the last sampling time. Treatment with anti-CRF, however, inhibited most of the ether-induced corticotropin response. The difference between the NRS- and the anti-CRF-treated groups was significant at 5 and 10 min ($P < 0.01$).

Cold Stress. The corticotropin response following exposure to cold stress is presented in Fig. 4. The NRS-treated group manifested an appropriate 4-fold elevation in plasma corticotropin concentrations at 5 min. In contrast, the effect

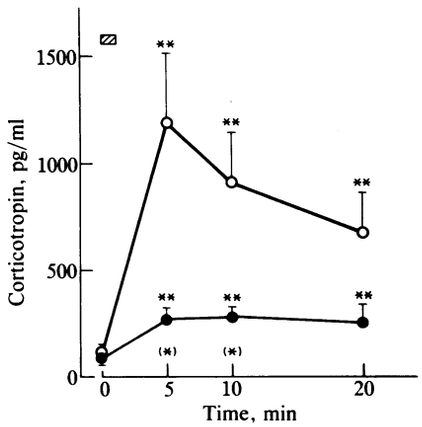

FIG. 3. Plasma corticotropin concentrations after 1-min exposure (hatched bar, top left) to ether vapor immediately after administration of NRS (○) or anti-CRF (●). Eight guinea pigs were used for each treatment. Significances (*, $P < 0.05$; **, $P < 0.01$) are defined in legend to Fig. 2.

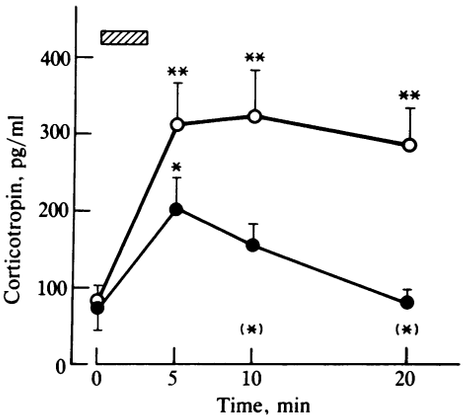

FIG. 4. Plasma corticotropin concentrations before and after 3-min cold stress (hatched bar, top left) in combination with NRS (○) or anti-CRF (●). Six guinea pigs were used for each treatment. Significances (*, $P < 0.05$; **, $P < 0.01$) are defined in legend to Fig. 2.

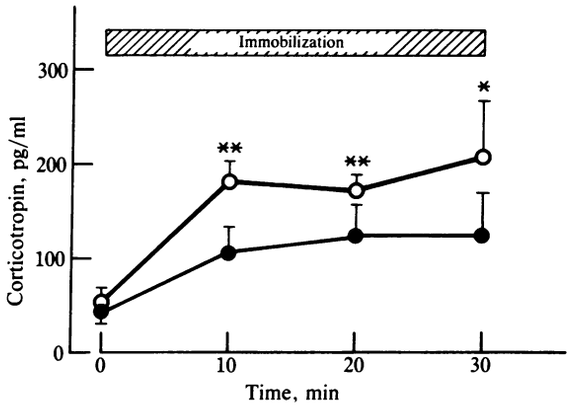

FIG. 6. Plasma corticotropin concentrations during immobilization in combination with NRS (○) or anti-CRF (●). Six guinea pigs were used for each treatment. Significances (*, $P < 0.05$; **, $P < 0.01$) are defined in legend to Fig. 2.

of this stress in the guinea pigs who had been given anti-CRF was blunted and more transient. The responses in the two groups differed at the 0.05 level of significance at 10 and 20 min.

Leg Fracture. The effects of unilateral leg fracture are shown in Fig. 5. In the NRS-treated group, the plasma corticotropin concentration increased gradually. Treatment with anti-CRF effectively maintained the corticotropin response at a consistently lower level throughout; however, a statistically significant difference between the two groups was evident only at 20 min after administration of the stress ($P < 0.01$).

Immobilization. The plasma corticotropin levels during immobilization are depicted in Fig. 6. Increased corticotropin secretion was statistically evident in the NRS-treated group ($P < 0.01$ at 10 and 20 min; $P < 0.05$ at 30 min). In the group treated with anti-CRF, none of the corticotropin values during immobilization stress differed significantly from control values.

DISCUSSION

The pituitary–adrenal axis can be activated by a bewilderingly wide variety of stressful stimuli, which, probably because of the significant psychologic component often present, have proven surprisingly difficult to define in objective terms and to characterize with precision (26–28). However, whatever classification is applied, the various stresses are likely to differ both qualitatively and quantitatively with respect to the extent of conjoint mediation by CRF, catecholamines, and arginine vasopressin as well as of susceptibility to steroid suppression (14, 16, 19, 20). They also differ in the patterns of hormonal response, both with respect to those "nonspecific" hormones that characteristically respond to many stimuli, such as epinephrine, norepinephrine, cortisol, growth hormone, and prolactin, as well as with respect to those hormonal functions in which the response is stressor-dependent (14, 16): for example, thyroid function, which is suppressed by some stresses but acutely activated by others, such as cold exposure (6, 15, 19). Indeed, available data suggests that stress evokes a multihormonal-patterned response that may be stress-specific.

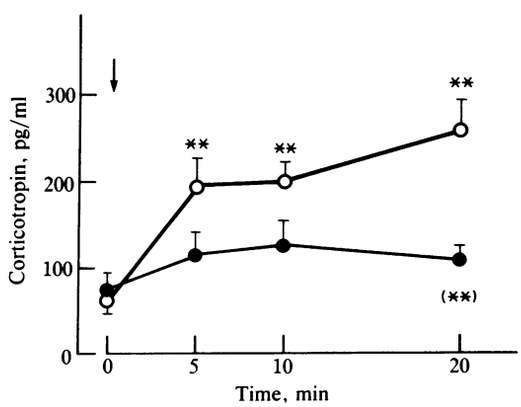

FIG. 5. Plasma corticotropin concentrations following trauma (arrow), in the form of unilateral leg fracture, in combination with NRS (○) or anti-CRF (●). Seven guinea pigs were used for each treatment. Significances (**, $P < 0.01$) are defined in legend to Fig. 2.

A potential first mediator of the stress response has emerged from the landmark studies of Vale and his colleagues (29, 30), who have established the structure of ovine and, more recently, rat CRF and have shown that these peptides are highly potent *in vitro* and *in vivo* in eliciting secretion of corticotropin and β-endorphin (29, 31). The parallel findings that CRF also stimulates motor activation, oxygen consumption, and prolonged elevations of plasma concentrations of epinephrine, norepinephrine, and glucose (32); the observations that CRF decreases grooming and food ingestion (33) and inhibits sexual receptivity in the female rat (34); and other findings of behavioral activation (35) are compatible with the concept that CRF plays a central role in coordinating the pathophysiological sequelae of stressful stimuli. Indeed, it was precisely to test this hypothesis that Vale and co-workers examined the effects of ether stress in the rat (4). The studies of Vale document the essential role of an endogenous CRF-like molecule, presumably CRF itself, in causing corticotropin secretion in this *in vivo* paradigm.

It has been shown that ether, even after a very short action, is a potent acute stressor that elicits a very rapid and dramatic increment in plasma corticotropin and corticosterone (5, 18). Moreover, although it has been suggested that ether activates the hypothalamic γ-aminobutyric acid system, which would then prevent any further response of the hypothalamic CRF system to stress (36), there is no decrease in the ability to maintain corticotropin levels during 2 hr of ether anesthesia.

However, the neural mediation of corticotropin secretion in response to ether appears to differ from that with other stresses in a fundamental sense. Thus, severely stressful systemic stimuli, such as *Escherichia coli* endotoxin (20), a large dose of formaldehyde, hemorrhage, and laparotomy, are thought to bypass the hypothalamus and possibly act di-

rectly on the pituitary or conceivably induce the release of a postulated "tissue CRF," since it has been found that there is a considerable store of CRF-like materials outside the brain (37, 38). In contrast, the adrenal response to most other stresses, such as severe cold, surgical trauma, and acoustic or photic stimuli, is eliminated by total disconnection of the hypothalamus from the rest of the brain. Although there is some evidence that other brain centers may be involved in the steroid inhibition of the corticotropin response to ether, so that the response to ether is probably modulated by other neural inputs, the response itself appears to require only an intact hypothalamus, as documented by Matsuda *et al.* (39) and further corroborated by Greer and Rockie (10) and Dunn and Critchlow (7). Indeed, recent evidence from several different experimental approaches (35, 40, 41) suggests that the paraventricular nuclei play a major role in mediating at least the early acute phase of the corticotropin secretion evoked by ether.

Nevertheless, these findings with ether cannot be reliably extrapolated to the general pathophysiology of the many varieties of stress without further study, particularly in light of the unique neural mediation of the response to ether stress (5, 10, 11, 18). Therefore, the present studies were undertaken to ascertain if CRF also had a physiological role in mediating the corticotropin response to other stresses, each of which has its own distinctive features: Severe cold is a classical acute and chronic stress stimulus (6, 15, 19). When subjected to chronic cold, animals appear to be sensitized to superimposed acute stresses, such as ether. Moreover, in contrast to most other stresses, such as immobilization, cold activates the hypothalamic–pituitary–thyroid axis and acutely raises pituitary cyclic GMP levels but does not increase pituitary cyclic AMP (16). The precise manner in which cold is administered is likely to be significant, in that mild cold stresses may activate only corticosterone secretion without affecting levels of other "stress" hormones (16). Conversely, the response to immobilization, which also may be mediated by non-neural pathways, has been shown to markedly increase pituitary cyclic AMP and prolactin but, interestingly, not cyclic GMP (16). The corticotropin response to trauma appears to depend on the ascending neural pathways, which cross in the midbrain and involve the contralateral pons (10, 11).

In each of the model stresses we have examined, there was evidence for a physiological role of CRF as shown by immunoneutralization with anti-CRF of the stress-induced increment in corticotropin release. The anti-CRF in these studies did not lower the base-line corticotropin level in any case, nor did the lower limit of corticotropin levels ever fall below the base line. The corticotropin responses to ether and cold were rapid and more short-lived than those observed with immobilization and trauma. However, anti-CRF administration produced a decrease of the increment in corticotropin induced by each model stress. Nevertheless, in all four cases, the inhibition observed was incomplete, with clear-cut residual secretion of corticotropin in animals treated with anti-CRF, a finding similar to that noted by the Riviers and Vale (4) for ether stress.

At a dilution of 1:300, the antiserum used in our studies was shown *in vitro* to precipitate >90% of [[^{131}I]iodotyrosine]oCRF. Even allowing liberally for dilution in the guinea pig blood volume and for the possibility of low interspecies crossreactivity, the antibody was used at a concentration far in excess of that theoretically required for complete immunoneutralization of all endogenous CRF. Nevertheless, we cannot fully exclude the possibility that our observations may merely reflect, in whole or in part, an inability of our antiserum raised against oCRF to neutralize the endogenous CRF of the guinea pig sufficiently rapidly or completely to fully prevent the corticotropin increase. However, our findings more likely reflect the contribution of additional mediators, notably catecholamines and vasopressin, of corticotropin release in response to these stresses, as has been shown for ether stress by Rivier and Vale (42). Indeed, their studies with CRF antiserum, a vasopressin antagonist, and the ganglionic blocker chlorisondamine suggest that a plurality of factors in addition to CRF modulate corticotropin secretion, at least as a response to ether stress (42), and support the involvement of factors other than CRF in the stress response in general. Moreover, the varying efficacy of the three inhibitors with time relative to application of stress underscores the complexity of the mechanisms involved in this activation.

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1. Guillemin, R. & Rosenberg, E. (1955) *Endocrinology* **57,** 599–607.
2. Saffran, M. & Schally, A. V. (1955) *Can. J. Biochem. Physiol.* **33,** 408–415.
3. Guillemin, R., Vargo, T., Rossier, J., Minick, S., Ling, N., Rivier, C., Vale, W. & Bloom, F. (1977) *Science* **197,** 1367–1369.
4. Rivier, C., Rivier, J. & Vale, W. (1982) *Science* **218,** 377–379.
5. Cook, D., Kendall, J. W., Monte, A., Greer, M. A. & Kramer, R. M. (1973) *Endocrinology* **93,** 1019–1024.
6. Daniels-Severs, A., Goodwin, A., Keil, L. C. & Vernikos-Danellis, J. (1973) *Pharmacology* **9,** 348–356.
7. Dunn, J. & Critchlow, V. (1969) *Brain Res.* **16,** 395–403.
8. Gibbs, F. P. (1969) *Am. J. Physiol.* **217,** 84–88.
9. Gibbs, F. P. (1969) *Am. J. Physiol.* **217,** 78–83.
10. Greer, M. A. & Rockie, C. (1968) *Endocrinology* **83,** 1247–1251.
11. Hedge, G. A., Van Ree, J. M. & Versteeg, D. H. G. (1976) *Neuroendocrinology* **21,** 236–246.
12. Kant, G. J., Bunnell, B. N., Mougey, E. H., Pennington, L. L. & Meyerhoff, J. L. (1983) *Pharmacol. Biochem. Behav.* **18,** 967–971.
13. Kant, G. J., Mougey, E. H., Pennington, L. L. & Meyerhoff, J. L. (1983) *Life Sci.* **33,** 2657–2663.
14. Kvetnansky, R., Kopin, I. J. & Saavedra, J. M. (1978) *Brain Res.* **155,** 387–390.
15. Langer, P., Foldes, O., Kvetnansky, R., Culman, J., Torba, T. & El Daher, F. (1983) *Exp. Clin. Endocrinol.* **82,** 51–60.
16. Lenox, R. H., Kant, G. J., Session, G. R., Pennington, L. L., Mougey, E. H. & Meyerhoff, J. L. (1980) *Neuroendocrinology* **30,** 300–308.
17. Saavedra, J. M. (1982) *Neuroendocrinology* **35,** 396–401.
18. Velluci, S. V. (1977) *Br. J. Pharmacol.* **60,** 601–605.
19. Vernikos, J., Dallman, M. F., Bonner, C., Katzen, A. & Shinsako, J. (1982) *Endocrinology* **110,** 413–420.
20. Yasuda, N. & Greer, M. A. (1978) *Endocrinology* **102,** 947–953.
21. Wehrenberg, W. B., Brazeau, P., Luben, R., Bohlen, P. & Guillemin, R. (1982) *Endocrinology* **111,** 2147–2148.
22. Hollander, C. S., Audhya, T., Russo, M., Passarelli, J., Nakane, T. & Schlesinger, D. S. (1983) *Endocrinology* **112,** 2206.
23. Hollander, C. S., Audhya, T., Nakane, T., Kanie, N., Kageyama, N., Kuwayama, A., Golbe, L. & Schlesinger, D. H. (1983) *Trans. Am. Assoc. Physicians* **96,** 122–130.
24. Orth, D. N., Jackson, R. V., Decherne, G. S., DeBold, C. R., Alexander, A. N., Island, D. P., Rivier, J., Rivier, C., Spiess, J. & Vale, W. (1983) *J. Clin. Invest.* **71,** 587–595.
25. Nicholson, W. E., Davis, D. R., Sherrell, B. J. & Orth, D. N. (1984) *Clin. Chem.* **30,** 259–265.
26. Martin, J. B., Reichlin, S. & Brown, G. M. (1977) *Clin. Neuroendocrinol.* **8,** 179–200.
27. Mason, J. W. (1975) *J. Hum. Stress* **1,** 6.

28. Mason, J. W. (1975) *J. Hum. Stress* **1,** 22.
29. Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* **213,** 1394–1397.
30. Spiess, J., Rivier, J., Rivier, C. & Vale, W. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6517–6521.
31. Rivier, C., Brownstein, M., Spiess, J., Rivier, J. & Vale, W. (1982) *Endocrinology* **110,** 272–278.
32. Brown, M. R., Fisher, L. A., Spiess, J., Rivier, C., Rivier, J. & Vale, W. (1982) *Endocrinology* **111,** 928–931.
33. Morley, J. E. & Levine, A. S. (1982) *Life Sci.* **31,** 1459–1464.
34. Sirinathsinghji, D. J. S., Rees, L. H., Rivier, J. & Vale, W. (1983) *Nature (London)* **305,** 232–235.
35. Vale, W., Rivier, C., Brown, M. R., Spiess, J., Koob, G., Swanson, L., Bilezikjian, L., Bloom, F. & Rivier, J. (1983) *Recent Prog. Horm. Res.* **39,** 245–270.
36. Manev, H. & Pericic, D. (1983) *Pharmacol. Biochem. Behav.* **18,** 847–850.
37. Brodish, A. (1963) *Endocrinology* **73,** 727–735.
38. Brodish, A. (1977) *Ann. N.Y. Acad. Sci.* **297,** 420–435.
39. Matsuda, K., Kendall, J. W., Duyck, C. & Greer, M. A. (1964) *Endocrinology* **75,** 981–985.
40. Tilders, F. J. H., Schipper, J., Lowry, P. J. & Vermes, I. (1982) *Regul. Pept.* **5,** 77–84.
41. Bruhn, T. O., Plotsky, P. M. & Vale, W. (1984) *Endocrinology* **114,** 57–62.
42. Rivier, C. & Vale, W. (1983) *Nature (London)* **305,** 325–327.