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Radioimmunoassay of CRF-Like Material in Rat Plasma: Validation of the Method (41656)

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Abstract. The availability of antibodies against the ovine corticotropin releasing factor (CRF), which cross-react with a CRF-like immunoreactivity (CRF-LI) in the rat, has enabled us to develop a radioimmunoassay (RIA) for rat CRF-LI in plasma and crude hypothalamic extracts. ^{125}I-Tyr CRF 1-41 was used as the tracer, and synthetic ovine CRF as the reference hormone. The precision profile of the assay indicates a high degree of reproducibility except for the lower dose range. The minimum detectable dose was 20 pg/tube. This assay can detect differences in plasma CRF-LI levels after various manipulations that simultaneously alter the ACTH levels in plasma. A wide range of CRF concentrations has been found in plasma of normal rats. Caution should be exercised in the interpretation of the values obtained since an ovine RIA system was used.

Isolation and structural characterization of a 41-amino acid peptide with CRF activity *in vivo* and *in vitro* was recently reported by Vale *et al.* (1, 2). The availability of synthetic CRF has allowed the production of specific antibodies and led to the immunocytochemical localization of CRF-LI in the central nervous system of several species, including man (3–5). These findings, and the reports on its biological activity in various *in vivo* and *in vitro* systems (6–9), support the view that this peptide might be the physiological corticotropin-releasing hormone.

However, a sensitive measurement technique, e.g., a radioimmunoassay, is required to further investigate the possible physiological role of this new hypothalamic peptide. To our knowledge, only a limited number of reports have appeared on CRF-LI in rat hypothalamic tissue (10, 11) and in plasma of pentobarbital-anaesthetized rats (12). We have previously reported the characterization of an anti-ovine CRF antibody by immunocytochemistry and RIA (13).

In this paper we describe the development of a specific RIA for CRF-LI in rat plasma and crude hypothalamic extracts, and its validation in various experimental conditions *in vivo*.

Materials and Methods. *Antibodies.* Immunization procedure and antibody characterization have already been reported in detail elsewhere (13). Briefly, synthetic ovine CRF 1-41 synthesized in our laboratories (7), was coupled to bovine serum albumin (BSA) with glutaraldehyde and used as an immunogen to produce antibodies in rabbits. The specificity of the raised antibodies was determined by agar-gel diffusion, absorption and double staining tests in the immunocytochemical system, and by cross-reactivity tests with other hypothalamic and pituitary hormones in the immunoassays.

Iodination. Since the native peptide lacks a tyrosine residue, synthetic Tyr°-CRF 1-41 (Peninsula, San Carlos, California) was used for labeling purposes. The iodination was carried out by the chloramine-T method. Twenty microliters of 0.25 *M* phosphate buffer, pH 7.4, and 500 μCi of ^{125}I-Na (Amersham, Illinois) were added to a polypropylene tube containing 4 μg of the peptide in 10 μl of 0.01 *N* HCl, followed by 60 μg chloramine-T in 10 μl of phosphate buffer. Forty seconds later a 10% solution of mercaptoethylamine (in the same phosphate buffer) was added to stop the reaction, and the mixture was diluted with 1 ml of human serum albumin (HSA, 5% in distilled water). A Sephadex G-50 column (1.5 × 30 cm, Pharmacia, Piscataway, N.J.) was used to separate labeled hormone from free iodine, using 2 *N* acetic acid containing 0.1%

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BSA as eluent buffer. Two distinct peaks were obtained, the first corresponding to the labeled hormone as determined by its immunoreactivity.

Radioimmunoassay procedure. The assay buffer was 0.5% BSA in 0.05 *M* phosphate–0.01 *M* NaCl, pH 7.6. The incubation mixture in the RIA tubes consisted of 0.1–0.2 ml buffer, 0.1 ml standard (range: 20–2560 pg/tube) or sample, and 0.1 ml of anti-CRF serum (SV 23 at a final dilution of 1:30,000). In order to correct for nonspecific effects, 0.1 ml of rat plasma or hypothalamic extract, treated with dextran-coated charcoal, was added to the standard curve tubes. Three hours later, 0.1 ml of tracer was added to all tubes (8000 cpm), and after an overnight incubation at 4°C, the bound and free fraction were separated by dextran–charcoal as previously described (13).

Sample processing. Rat blood was collected from the trunk in a tube containing EDTA (1 mg/ml) after decapitation and centrifuged at 4°C. The plasma was divided among three to four tubes and samples were kept at −56°C. The hypothalamic tissue was dissected and immediately placed on dry ice, and either extracted with 2 *N* acetic acid and lyophilized or kept at −20°C until extracted. These samples were dissolved in the assay buffer and tested for CRF in order of increasing dilutions.

Animals. Sprague–Dawley rats weighing 300–400 g were used. Animals were allowed to adapt for 3–7 days after arrival. They were placed in a constant-temperature (24 ± 2°C), light-controlled room (12 hr light–12 hr dark schedule) and allowed rat standard diet and tap water *ad libitum.*

Chlorpromazine – morphine – nembutal (CPZ–M–N) anaesthetized rats. Male rats were prepared as described by Arimura *et al.* (14). One hour after Nembutal administration they were decapitated and blood was collected from the trunk for CRF and ACTH determination.

Lesioned animals. Male rats were anaesthetized with pentobarbital (40 mg/kg body weight, ip). An anterolateral (AL) cut around the medial basal hypothalamus (MBH) or a sham operation was performed in a stereotaxic frame with a knife of both 1.8 mm height and radius (15). The cut, with a forward convexity, was placed just behind the optic chiasm and extended backward for 2.8 mm on both sides, 1.8 mm lateral to the midline. Posterolateral and posterior connections of the MBH, as well as neuronal and vascular connections between the MBH and the pituitary, were not disturbed. Sham operations were performed by lowering the knife into the brain at the appropriate starting coordinates, and then removing it without making any lesion. The lesioned animals developed diabetes insipidus but did not gain weight.

Fifteen days later, rats were stressed with ether according to Cook *et al.* (16) and killed 5 min after the initiation of the stress. One group of the lesioned animals was killed directly without ether stress to serve as controls. Blood was collected from the trunk and processed as described above. The pituitaries and the adrenal glands were quickly removed and weighed. The brains were kept in a 4% formaldehyde solution. Later, they were embedded in paraffin, sectioned serially, and stained with methylene blue for histological evaluation.

ACTH radioimmunoassay. Anti-pig ACTH and human ACTH (1–39), used both as a standard and for iodination, were provided by the National Pituitary Agency. ACTH iodination was carried out as described by Kao *et al.* (17) using a CM-25 ion exchanger column to further purify the labeled hormone. The assay was done in a similar fashion to that reported previously (7): a 0.01 *M* phosphate–0.14 *M* NaCl–0.025 *M* EDTA buffer, containing 0.35% HSA, 4% Trasylol, and 0.4% mercaptoethanol, was used as assay diluent. To the RIA tube were added the standards or the samples diluted with the assay buffer, and the anti-pig ACTH (1:12500 final). Appropriate aliquots of rat plasma stripped of ACTH with dextran-coated charcoal were included in the standard curve tubes. Two hours later, 0.1 ml of labeled hormone (10,000 cpm) was added. After an 18-hr incubation period at 4°C, dextran–charcoal was used to separate the free from the bound fraction.

The intraassay coefficient of variation (CV) was 5.7 and 4.7% for a dose of 500 and 125 pg/tube, respectively, and the interassay CV was 5.2 and 2.6% for the same respective doses. The sensitivity of the assay was 5 pg/tube.

Statistics. The data were compared using a Student's *t* test for the CPZ–M–N experiment and a Duncan's test for the lesions experiments. The values are given as mean ± SE.

Levels below the sensitivity of the assay were considered as 200 pg/ml for statistical purposes.

Results. *Iodination.* The mean incorporation of iodine into the Tyr°-CRF molecule was 50%, although with freshly prepared solutions of peptide, the incorporation obtained was more than 85%. The calculated mean specific activity was 75 μCi/μg of peptide, but when fresh solutions were used this could be increased up to 110 μCi/μg. On the basis of these results we recommend the use of peptide solutions prepared just before iodination takes place.

Assay performance. Figure 1 shows three standard curves obtained: (I) using assay buffer as diluent, (II) assay buffer + 100 μl charcoal-treated rat plasma, or (III) assay buffer + 100 μl charcoal-treated rat hypothalamic extract. A 3–5% increase in the "nonspecific binding" (NSB) (B/T values) was found when using charcoal-treated plasma or hypothalamic extracts. This increase in NSB values was also seen in the samples (not treated with charcoal), suggesting the existence of a nonspecific interference in the high-molecular weight components of the sample (18).

A 10–15% total binding value was determined to give the greatest sensitivity within the assay conditions used. A minimum detectable dose of 20 pg/tube was found using a paired t test ($P < 0.001$), although a theoretical sensitivity of 15 pg/tube is possible using the lower limit of the 95% confidence limits of the buffer-control tubes.

Increasing dilutions of hypothalamic extracts showed linearly decreased CRF-LI content, as can be seen in Fig. 2. A 95% recovery was achieved for the extraction procedure when synthetic CRF was added to the tissue prior to extraction. We observed a 200% decrease in the CRF-LI hypothalamic content when the hypothalami were stored for more than 3 weeks at −20°C before extractions.

When synthetic ovine CRF was added to a pool of rat plasma and diluted in increasing order, it gave a B/B_0 vs dose relationship parallel to the standard curves (see Fig. 1). The recovery of standard CRF, added to rat plasma and incubated at room temperature for 30 min, was 70%. The mean plasma CRF-LI found in normal rats ($N = 90$, both sexes) was 270 ± 35 pg/ml (mean ± SE), with a range of <200–1300 pg/ml. Although we have not ruled out a possible nonspecific interference of the organic-extractable plasma phase in the RIA system, an extraction procedure in C-18 Sep-Pak cartridges (Waters Assoc., Mass.), using trifluoroacetic acid 0.1%: acetonitrile 20:80 (vol/vol) as organic eluent, did not lower the values of CRF-LI in plasma as measured by RIA.

A precision profile (19) based on the intra- and interassay CV for five different doses of

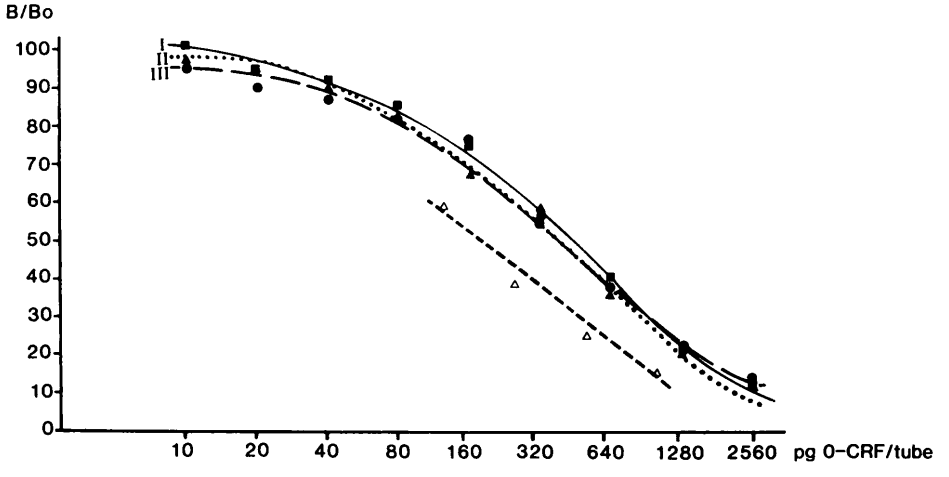

FIG. 1. Standard curves of ovine CRF using assay buffer (I), assay + charcoal-treated rat plasma (II), or assay buffer + charcoal-treated rat hypothalamic extract (III) as eluent. Dashed line: synthetic ovine CRF added to a rat plasma pool and diluted in increasing order showed parallelism to the standard curves.

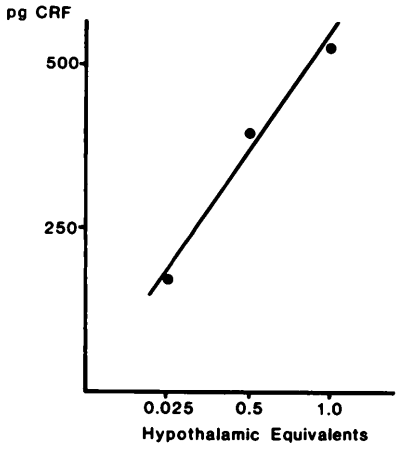

FIG. 2. Relationship between tissue equivalents and CRF-LI dose of a pooled rat hypothalamic extract as determined by RIA.

hormone is given in Fig. 3. Both patterns showed good agreement. On the other hand, as expected, when using nonpurified antibody preparations, Scatchard analysis of the antigen–antibody interaction showed more than one saturable binding site, confirming the existence of more than one population of antibodies with different affinities for the ligand.

Plasma CRF-LI in CPZ–M–N-treated rats. Figure 4 illustrates the CRF-LI and ACTH plasma levels in CPZ–M–N-treated rats and their respective controls. A parallel decrease of ACTH and CRF-LI in the injected rats can be seen as compared to the untreated group.

Response to ether stress in hypothalamic-lesioned rats. Figure 5 shows that the stress-induced elevation of ACTH and CRF-LI plasma was blocked in the lesioned animals. CRF-LI levels similar to those in the non-stressed group were obtained. Only animals with correctly placed lesions, as checked by histology and immunohistology of brain sections, were used.

A slight, but not significant, decrease in the weight of the adrenal glands was observed in the lesioned animals as compared to the controls: 22.1 ± 1.1 mg for control vs 18.5 ± 0.4 mg for treated (mean ± SE), while the pituitaries remained unchanged: 11.6 ± 0.2 vs 10.1 ± 0.1 mg, respectively.

Discussion. In our preliminary report (13), we described the use of a Tyr^{35}-CRF 36-41 synthetic analog of ovine CRF as labeled hormone, which gave standard curves parallel to those obtained when using the Tyr^{0}-CRF 1-41 analog. Since it is theoretically more de-

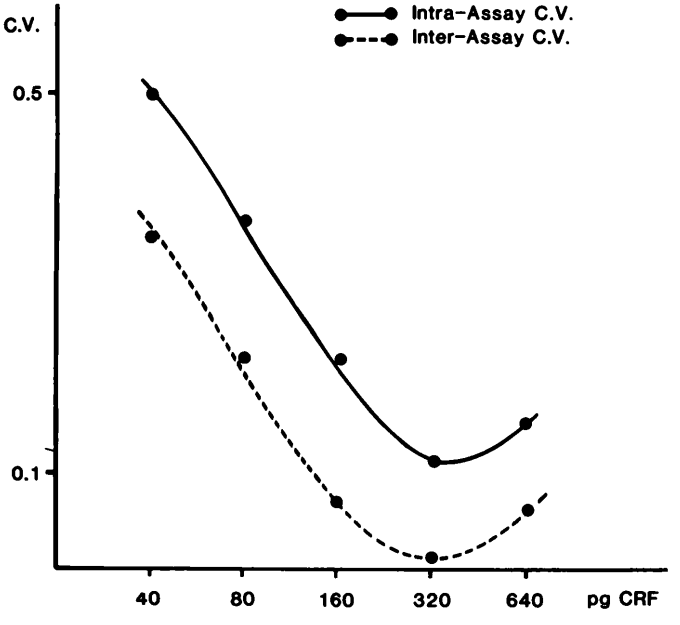

FIG. 3. Precision profile of the RIA using the inter- (dashed line) and intraassay (continuous line) CV for five different doses of hormone.

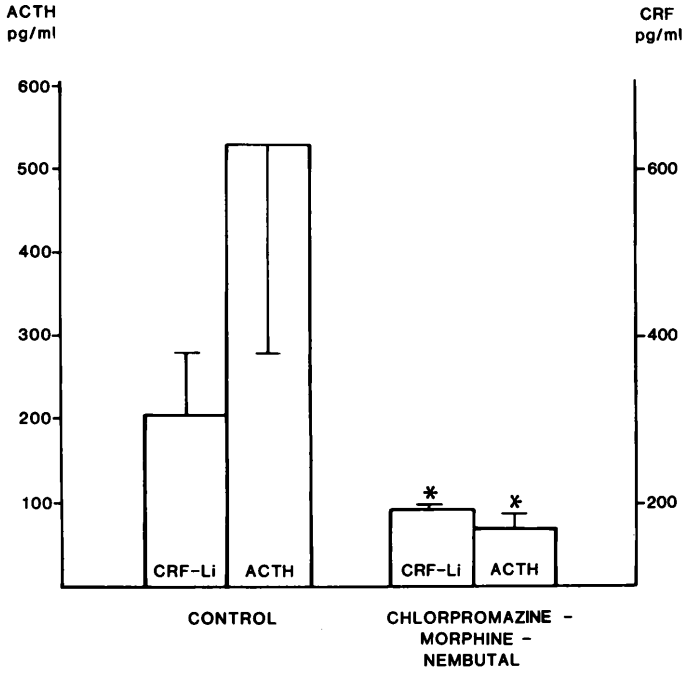

FIG. 4. ACTH and CRF-LI plasma levels in control (N = 4) and CPZ-M-N-treated rats (N = 6). *$P < 0.05$.

sirable to use a system in which both the labeled and the cold hormone react with the antibody in a similar way, we have developed an RIA for CRF using the Tyr° analog as tracer and synthetic ovine CRF as reference hormone. The sequence of the ovine CRF is

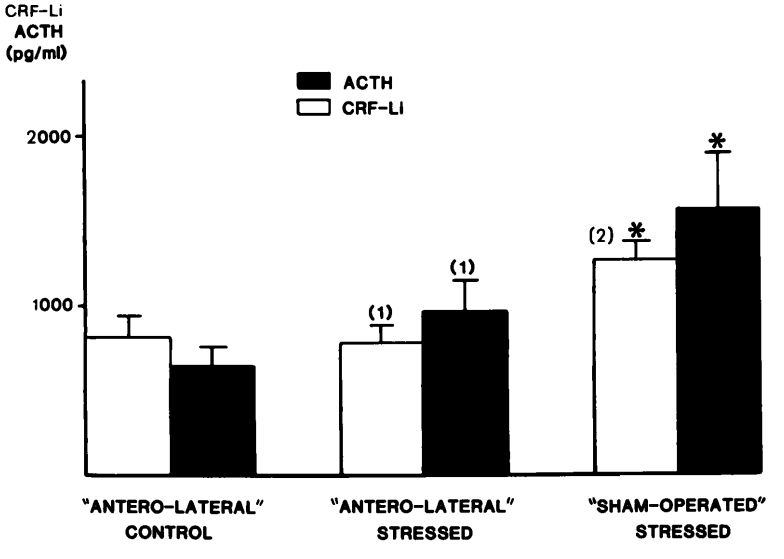

FIG. 5. ACTH and CRF-LI plasma levels after ether stress in rats with hypothalamic lesions (N = 7, AL) and sham operated (N = 6, sham). Eight lesioned animals served as nonstressed control group. (1) n.s. vs control; *$P < 0.05$ vs control. (2) $P < 0.05$ vs AL stressed.

expected to show more similarity to the native rat CRF than to the heptapeptide analog (20, 21).

The standard curves to which aliquots of either CRF-stripped plasma or hypothalamic extract were added (in order to obtain a similar "chemical milieu" to the sample), and the curves with assay buffer only, were parallel. Similarly, linear dose vs dilution-factor relationship, and parallelism with the standard curves at various dilutions of CRF added to plasma (or hypothalamic extract), were also obtained. However, these parameters do not completely validate the system.

The precision profile of the assay indicates a high degree of reproducibility as inferred from the close parallelism of the inter- and intraassay CV patterns. However, the higher CV present in the lower dose range shows some lack of precision of the method, even though the sensitivity of the assay was 20 pg or less. On the other hand, we have overcome this problem in practice, as shown by the clear difference in the values obtained for each group of animals studied. We are presently conducting further studies to solve this lack of precision in the lower range of the assay.

Another approach to validate the assay was to use animals under different conditions, which we assumed would show different CRF levels. Since the CPZ–M–N assay was first developed (14), it has been surmised that the decrease in ACTH and corticosterone levels is caused by a blockade of CRF release through a central action of the drugs employed. However, to our knowledge this is the first report of a decrease in CRF-LI parallel to a fall in plasma ACTH levels. This reinforces a primary role for CRF in the hypophyseal–adrenal response to pharmacological manipulations. Lesion-induced blockade of the CRF response to ether stress observed in the AL-deafferented rats suggests a disruption of the neurotransductory mechanism, which triggers the increase in the pituitary–adrenal output. This blockade could be due to a depletion of the CRF-containing terminals in the median eminence. These findings are in good agreement with the results of Makara *et al.* (21) using an ACTH bioassay and Merchenthaler *et al.* (unpublished observations) using CRF immunocytochemistry.

The low levels in hypothalamus found by us, 16.5 ng/g wet tissue (approximately 500 pg/hypothalamus), compare well with values of 12–18 ng/g reported by others (10, 23), but not with levels found for other hypothalamic hormones (10). We have observed a wide range of concentrations of plasma CRF-LI in normal rats, from values below the sensitivity of the assay up to 1300 pg/ml. This variation could possibly be explained by the existence of a pulsatile release of CRF. A circadian pattern in the content of hypothalamic CRF-LI has been reported (23).

In a recent report, Gibbs and Vale (12) stated that CRF-LI levels in extracted plasma of pentobarbital-anaesthetized male rats were either $\leqslant$27.5 p*M* or undetectable by their RIA. The concentration of 27.5 p*M* is in close agreement with values obtained by us, considering that their levels were admittedly underestimated by a factor of two (12).

On the other hand, plasma CRF levels are similar to those found for somatostatin (24). This hormone also has peripheral sources of secretion. Since CRF-LI has been found in the pancreas and stomach (I. Merchenthaler, unpublished observations), there is the possibility of a peripheral component of CRF-LI in the blood.

Rat CRF has not been characterized, and it is not yet available for use as a standard and for antibody production. Thus, the plasma CRF levels reported here should be considered as estimates of relative changes in CRF-LI concentrations rather than absolute values of rat CRF in plasma, since the degree of cross-reactivity with the antiovine CRF has not yet been determined.

The levels reported herein for rat ACTH are somewhat high. A possible explanation is that the RIA system used is heterologous, and both the antibody and the reference hormones were obtained from species other than the rat. Increased ACTH values have also been observed by others when using heterologous assays. Thus, ACTH levels in normal rats were reported to be 500 pg/ml (10) and in ether-stressed rats, 2000 pg/ml (16). Consequently, the values given should be taken only as relative variations.

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1. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science **213**:1394–1397, 1981.
2. Rivier C, Brownstein M, Spiess J, Rivier J, Vale W. In vivo corticotropin-releasing factor-induced secretion of adrenocorticotropin, beta-endorphin, and corticosterone. Endocrinology **110**:272–278, 1982.
3. Merchenthaler I, Vigh S, Petrusz P, Schally AV. Immunocytochemical localization of corticotropin releasing factor (CRF) in the rat brain. Amer J Anat **165**:385–396, 1982.
4. Paull WK, Sholler J, Arimura A, Meyers CA, Chang JK, Chang D, Shimizu M. Immunocytochemical localization of CRF in the ovine hypothalamus. Peptides **1**:183–191, 1982.
5. Bugnon C, Fellman D, Bresson J-L, Clavequin MC. Etude immunocytochimique de l'ontogenese du systeme neuroglandulaire a CRF chez l'homme. CR Acad Sci Paris **294**:491–496, 1982.
6. Turkelson CM, Arimura A, Culler MD, Fishback JB, Groot K, Kanda M, Luciano M, Thomas CR, Chang D, Chang JK, Shimizu M. In vivo and in vitro release of ACTH by synthetic CRF. Peptides **2**:425–429, 1981.
7. Sueiras-Diaz J, Coy DH, Vigh S, Redding TW, Huang W-Y, Torres-Aleman I, Schally AV. Synthesis and biological properties of ovine corticotropin-releasing factor (CRF). Life Sci **31**:429–435, 1982.
8. Grossman A, Nieuwenhuyzen-Kruseman AC, Perry L, Tomlin S, Schally AV, Coy DH, Rees LH, Comaru-Schally AM, Besser GM. New hypothalamic hormone, corticotropin-releasing factor, specifically stimulates the release of adrenocorticotropic hormone and cortisol in man. Lancet I, April 24:921–922, 1982.
9. Schulte HM, Chrousos GP, Oldfield EH, Gold PW, Cutler GB, Loriaux DL. The effects of corticotropin releasing factor on the anterior pituitary function of stalk-sectioned cynomolgus macaques: dose response of cortisol secretion. J Clin Endocrinol Metab **55**:810–812, 1982.
10. Moldow RL, Fischman AJ, Hypothalamic CRF-like immunoreactivity in the rat after hypophysectomy and adrenalectomy. Peptides **1**:143–147, 1982.
11. Moldow RL, Fischman AJ. Radioimmunoassay of CRF-like immunoreactivity in rat hypothalamus. Peptides **3**:37–39, 1982.
12. Gibbs DM, Vale W. Presence of corticotropin releasing factor-like immunoreactivity in hypophysial portal blood. Endocrinology **111**:1418–1420, 1982.
13. Vigh S, Merchenthaler I, Torres-Aleman I, Sueiras-Diaz J, Coy DH, Carter WH, Petrusz P, Schally AV. Corticotropin releasing factor (CRF): Immunocytochemical localization and radioimmunoassay (RIA). Life Sci **31**:2441–2448, 1982.
14. Arimura A, Saito T, Schally AV. Assays for corticotropin-releasing factor (CRF) using rats treated with morphine, chlorpromazine, dexametasone, and nembutal. Endocrinology **81**:235–245, 1967.
15. Halasz B, Pupp L. Hormone secretion of the anterior pituitary gland after physical interruption of all nervous pathways to the hypophysiotropic area. Endocrinology **77**:553–562, 1965.
16. Cook DM, Kendall JW, Greer MA, Kramer RM. The effect of acute or chronic ether stress on plasma ACTH concentration in the rat. Endocrinology **93**:1019–1024, 1973.
17. Kao PC, Jiang N-S, Carpenter PC. Human corticotropin (ACTH) radioimmunoassay with synthetic 1–24 ACTH. Clin Chem **25**:1267–1273, 1979.
18. Bryant MG. Plasma artifacts in the radioimmunoassay. In: Bloom SR, ed. Gut Hormones. Edinburgh, Churchill–Livingstone, p120, 1978.
19. Ekins R. The precision profile: its use in RIA assessment and design. Q Ligand **4**:33–44, 1981.
20. Hashimoto K, Ohno N, Murakami K, Hattori T, Aoki Y, Takahara J. Characterization of rat hypothalamic CRF on high performance liquid chromatography with synthetic ovine CRF as a marker. In: Abstracts, 64th Annual Meeting of the Endocrine Society, San Francisco, p213, 1982.
21. Fischman AJ, Moldow RL. Extrahypothalamic distribution of CRF-like immunoreactivity in the rat brain. Peptides **1**:149–153, 1982.
22. Makara GB, Karteszi M, Kiss JZ, Palkovits M, Rappay G, Stark E. Corticoliberin (CRF) activity in selected hypothalamic regions in rats with anterolateral hypothalamic cuts. Neuropeptides **2**:287–293, 1982.
23. Moldow RL, Fischman AJ. Physiological changes in rat hypothalamic CRF: circadian, stress and steroid suppression. Peptides **3**:837–840, 1982.
24. Engelhardt W, Schwille PO. Development of a sensitive somatostatin radioimmunoassay and its application to plasma of stressed and non-stressed rats. Horm Metab Res **13**:318–323, 1981.

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