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Distribution of corticotropin-releasing factor receptors in primate brain

(neuropeptide/adenylate cyclase/limbic system/stress)

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ABSTRACT **The distribution and properties of receptors for corticotropin-releasing factor (CRF) were analyzed in the brain of cynomolgus monkeys. Binding of [^{125}I]tyrosine-labeled ovine CRF to frontal cortex and amygdala membrane-rich fractions was saturable, specific, and time- and temperature-dependent, reaching equilibrium in 30 min at 23°C. Scatchard analysis of the binding data indicated one class of high-affinity sites with a K_d of 1 nM and a concentration of 125 fmol/mg (≈30% of the receptor number in monkey anterior pituitary membranes). As in the rat pituitary and brain, CRF receptors in monkey cerebral cortex and amygdala were coupled to adenylate cyclase. Autoradiographic analysis of specific CRF binding in brain sections revealed that the receptors were widely distributed in the cerebral cortex and limbic system. Receptor density was highest in the pars tuberalis of the pituitary and throughout the cerebral cortex, specifically in the prefrontal, frontal, orbital, cingulate, insular, and temporal areas, and in the cerebellar cortex. A very high binding density was also present in the hippocampus, mainly in the dentate gyrus, and in the arcuate nucleus and nucleus tuberis lateralis. A high binding density was present in the amygdaloid complex and mamillary bodies, olfactory tubercle, and medial portion of the dorsomedial nucleus of the thalamus. A moderate binding density was found in the nucleus accumbens, claustrum, caudate-putamen, paraventricular and posterior lateral nuclei of the thalamus, inferior colliculus, and dorsal parabrachial nucleus. A low binding density was present in the superior colliculus, locus coeruleus, substantia gelatinosa, preoptic area, septal area, and bed nucleus of the stria terminalis. These data demonstrate that receptors for CRF are present within the primate brain at areas related to the central control of visceral function and behavior, suggesting that brain CRF may serve as a neurotransmitter in the coordination of endocrine and neural mechanisms involved in the response to stress.**

In addition to its role as a major regulator of corticotropin (ACTH) release from the anterior pituitary (1), corticotropin-releasing factor (CRF) also exerts direct actions within the central nervous system in several animal species. Intracerebroventricular administration of CRF in experimental animals results in altered behavior and activates the hypothalamic-pituitary adrenal axis and the sympathetic nervous system, similar to the changes observed during stress (1–4). Immunoreactive CRF has been found in several extrahypothalamic sites in the brain, including many parts of the limbic system and centers known to control the autonomic nervous system (1, 5–7). These findings suggest that the peptide acts as a neurotransmitter in the brain to modulate the integrated responses to stress. This view is supported by the demonstration that CRF receptors are present in the rat brain, with a distribution corresponding to the components of the limbic system and cerebral cortex (8). There is evidence indicating that CRF may also be involved in the control of central nervous system function in primates including man. In this regard, changes in behavior and visceral function have been observed in the monkey after intracerebroventricular administration of CRF (4). Also, many patients with depression exhibit hyperactivity of the pituitary-adrenal axis, which could arise from a central disorder that is accompanied by increased secretion of CRF (9). To determine the sites at which CRF exerts its putative central actions in the primate, we have characterized CRF receptors and analyzed their anatomical distribution in the monkey brain.

MATERIALS AND METHODS

Ovine CRF (oCRF) and [Tyr]oCRF were synthesized as described (10). [[^{125}I]Tyr]oCRF was prepared by using the Iodogen technique with 2 μg of [Tyr]oCRF and 1 mCi of Na^{125}I, followed by HPLC purification on a C-3 reverse-phase column (Beckman) using a gradient (30:70 to 80:20) of acetonitrile/1% trifluoroacetic acid in 0.1 M ammonium acetate. The product had a specific activity of 250–280 μCi/μg and a maximum binding capacity to an excess of rat pituitary membrane protein of 30% (8). The brains of two cynomolgus monkeys (*Macaca fascicularis*) were used for these experiments. The animals were sacrificed with an overdose of pentobarbital, and the brains were immediately removed and sectioned. A parasaggital portion comprising approximately one-half of a hemisphere was placed in ice-cold 10 mM sodium/potassium phosphate/140 mM NaCl, pH 7.4, for the CRF binding assay and measurement of adenylate cyclase activity. The remaining tissue was frozen in 2-methylbutane at −40°C for autoradiographic analysis of CRF binding sites. Characterization of the brain binding sites was performed in 30,000 × *g* membrane-rich particles from the cingulate cortex, amygdala, and hippocampus, areas found to have a high concentration of receptors in preliminary autoradiographic experiments. For the binding assay, 200–300 μg of membrane protein was incubated at 22°C for 60 min with 0.2 nM [[^{125}I]Tyr]oCRF in 300 μl of 50 mM Tris·HCl (pH 7.4) containing 5 mM $MgCl_2$, 2 mM EGTA, 100 kallikrein inhibitor units of aprotinin, 1 mM dithiothreitol, and 0.1% bovine serum albumin in 1.5-ml polystyrene Microfuge tubes. Then, 7.5% polyethylene glycol in 50 mM Tris·HCl, pH 7.4, was added, and tubes were centrifuged at 10,000 × *g* for 3 min.

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Abbreviations: CRF, corticotropin-releasing factor; oCRF, ovine CRF.

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The pellet was washed with 1 ml of polyethylene glycol solution and the tip of the tube was severed and analyzed for bound radioactivity in a γ spectrometer. Nonspecific binding determined in the presence of 1 μM oCRF was <2% of the total radioactivity added.

For autoradiographic mapping of CRF binding, 20-μm frozen sections were processed as described (8). The slide-mounted sections were incubated for 15 min at 20°C in 50 mM Tris·HCl (pH 7.4) containing 5 mM $MgCl_2$, 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 140 mM NaCl, and 0.1% bovine serum albumin. The slides were then washed twice with 50 mM Tris·HCl buffer containing 2 mM EGTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM PMSF, 0.1% bovine serum albumin, and 5 mM $MnCl_2$; transferred to slidemailers (Curtis Matheson Scientific, Houston, TX) containing 8 ml of the same buffer containing aprotinin at 100 kallikrein inhibitory units, 1 mM dithiothreitol, and 0.2 nM [[^{125}I]Tyr]oCRF (300,000 cpm/ml); and incubated for 60 min at 22°C. Nonspecific binding was determined in the presence of 1 μM unlabeled oCRF. Incubation was terminated by four 1-min washes in ice-cold buffer and the slides were rapidly dried under cold air, fixed with formaldehyde vapor, and exposed to LKB Ultrofilm for 4 days at 4°C.

The films were processed and relative grain density was quantitated by computerized densitometry with color-coded image analysis (11).

Adenylate cyclase was measured by the conversion of [^{32}P]ATP to [^{32}P]cAMP as described (10, 12).

RESULTS

Properties of [Tyr]oCRF Binding Sites. In membrane-rich particles from the cingulate cortex, amygdala, and hippocampus, [[^{125}I]Tyr]oCRF bound to a single set of high-affinity sites with K_d values of 2.0 ± 0.2, 3.5 ± 0.3, and 1.9 ± 0.2 nM (mean ± SD, n = 2) and concentrations of 138 ± 9, 150 ± 7, and 47 ± 11 fmol/mg, respectively (Fig. 1). Binding of [[^{125}I]Tyr]oCRF to brain membranes was displaced by CRF peptides with ID_{50} values of 3 nM for oCRF, 1.2 nM for rat CRF, and 0.9 μM for the oCRF-(15–41) fragment. No displacement of the bound tracer was observed in the presence of the unrelated peptides, angiotensin II and arginine vasopressin, at concentrations up to 1 μM.

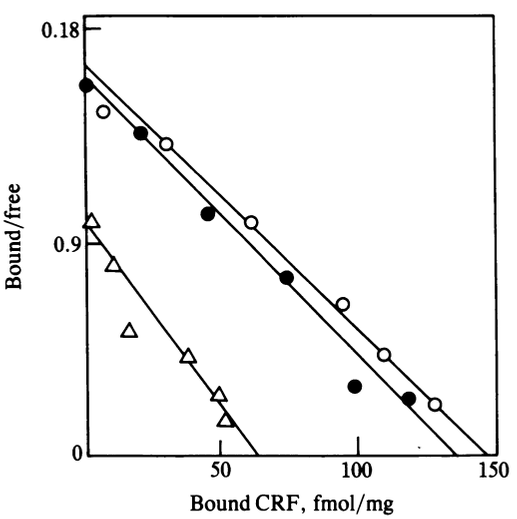

FIG. 1. Scatchard analyses of the binding of [[^{125}I]Tyr]oCRF to amygdala (○), cingulate cortex (●), and hippocampal (△) membrane-rich particles. Results represent means of duplicate determinations in one of two similar experiments.

Effect of CRF on Adenylate Cyclase Activity. CRF significantly increased adenylate cyclase activity in brain membranes from two regions that contain high concentrations of CRF receptors. CRF increased [^{32}P]cAMP formation from a basal value of 342 ± 20 pmol/mg per min to a value of 497 ± 15 pmol/mg per min with an ED_{50} of 0.5 μM in orbital cortex membranes and from 521 ± 32 to 692 ± 20 pmol mg per min with an ED_{50} of 0.42 μM in amygdala membranes (mean ± SD, n = 2). No effect of CRF on adenylate cyclase activity was observed in corpus callosum membranes lacking CRF receptors.

Autoradiographic Mapping of CRF Receptors in Monkey Brain. In the monkey brain, CRF receptors were located in the neocortex, several components of the limbic system, and the cerebellum, similar to the distribution of CRF receptors in the rat brain (Fig. 2 and Table 1). CRF receptors were found throughout the brain cortex, with markedly higher binding in the orbital (Fig. 2 *A–C*), prefrontal, frontal (Fig. 2*A*), insular (Fig. 2 *A–E*), temporal (Fig. 2 *B–E*), and cingulate areas (Fig. 2 *A–E*). In most cortical areas the receptors were confined to layers I and II, but in the frontal, orbital, insular, and temporal areas, the inner layers of the cortex were also labeled.

The hippocampus also contained a high concentration of CRF receptors with predominant labeling in the dentate gyrus (Fig. 2 *E* and *F*). Within extracortical areas, very high concentrations of receptors (optical densities > 450) were observed in the arcuate nucleus (Fig. 2*E*) and the nucleus tuberis lateralis. High concentrations of CRF receptors (optical densities of 350–450) were found in the amygdaloid complex (Fig. 2 *C* and *D*), mamillary bodies (Fig. 2*E*), olfactory tubercle (Fig. 2*B*), and the medial portion of the dorsomedial thalamic nucleus (Fig. 2*E*).

Moderate concentrations of receptors (optical densities of 200–350) were found in the nucleus accumbens (Fig. 2*B*), caudate-putamen (Fig. 2 *A–D*), claustrum (Fig. 2 *B* and *C*), paraventricular nucleus of the thalamus (Fig. 2*D*), posterior lateral nucleus of the thalamus (Fig. 2*F*), inferior colliculus (Fig. 2*G*), and dorsal parabrachial nucleus (Fig. 2*H*). Low concentrations of receptors (optical densities < 200) were found in the lateral septal nuclei, septal area, bed nucleus of the stria terminalis (Fig. 2*C*), anterior ventricular nucleus thalamus, preoptic area (Fig. 2*C*), lateral geniculate nucleus (Fig. 2*F*), superior colliculus (Fig. 2*F*), substantia nigra, and substantia gelatinosa.

The cerebellum contained a high concentration of CRF receptors that were distributed throughout with predominant labeling in the granular layer (Fig. 2 *G* and *H*).

DISCUSSION

These studies in the monkey show that specific receptors for CRF are present in the primate brain, with a predominant distribution that includes several areas associated with the control of behavior, peripheral endocrine responses, and the autonomic nervous system. The characteristics of the binding sites in the monkey brain are similar to those described for the rat pituitary (13) and brain (8), with high affinity and specificity for CRF and related peptides. As in the rat (14), the CRF binding sites are coupled to adenylate cyclase activity, suggesting that they are true receptors through which the peptide regulates neural function.

In the monkey, the receptor distribution in the brain cortex and limbic system-related structures resembles that in the rat brain. Whereas in the rat the receptors were evenly distributed throughout the cortex, in the monkey there were marked differences between the cortical areas. The highest binding was in the prefrontal, orbital, and insular cortices (15), regions of relatively late phylogenetic acquisition that are

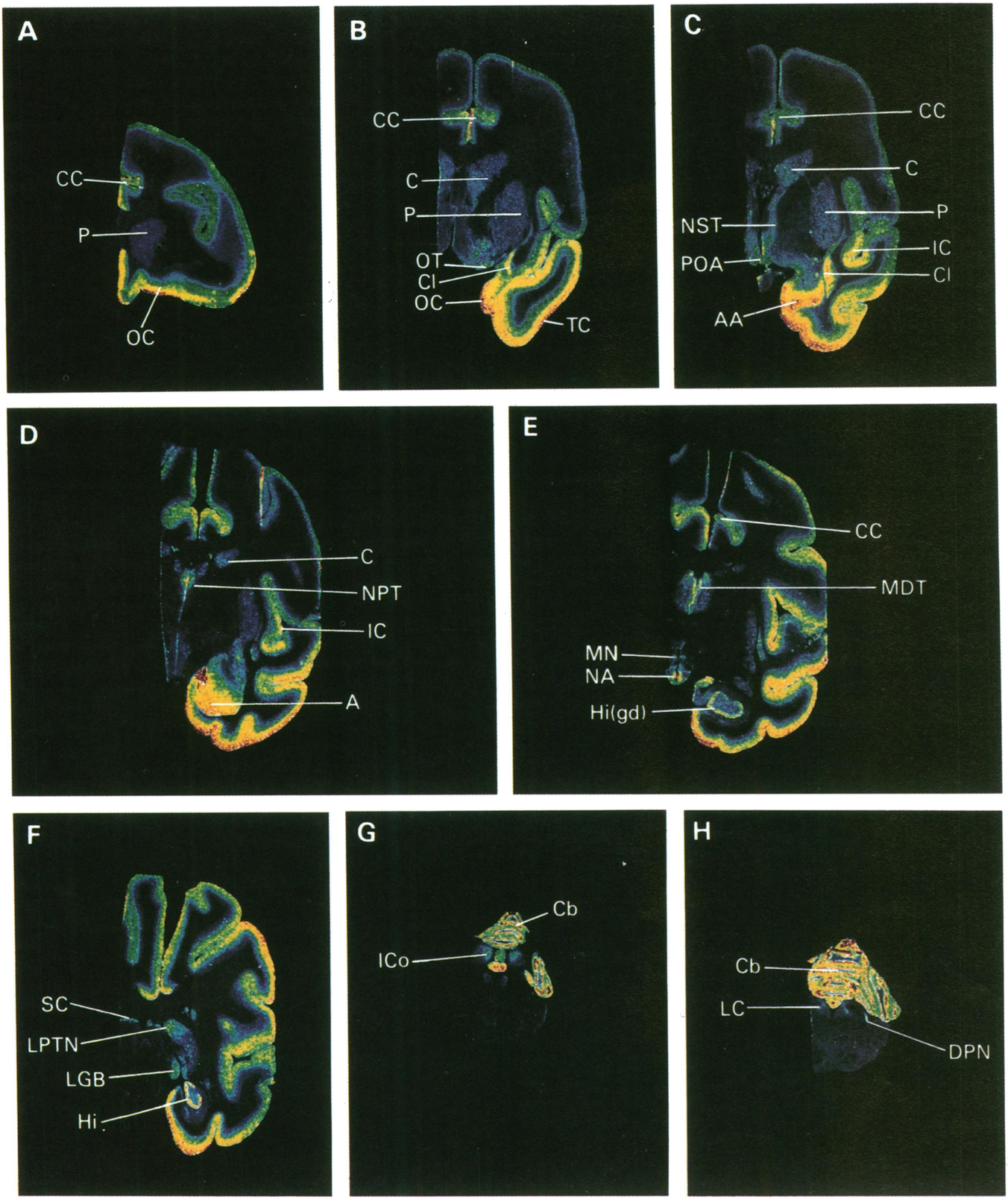

FIG. 2. Color-coded image of [[^{125}I]Tyr]oCRF autoradiographs of representative coronal sections from rostral to caudal of monkey brain. Areas of very high density are shown in red, and densities decrease through yellow, green, and light blue. Dark blue and black correspond to nonspecific background. CC, cingulate cortex; P, putamen; OC, orbital cortex; C, caudate; OT, olfactory tubercle; Cl, claustrum; TC, temporal cortex; NST, nucleus of stria terminalis; IC, insular cortex; POA, preoptic area; AA, anterior amygdala; NPT, paraventricular nucleus of thalamus; A, amygdala; MDT, medial dorsal thalamus; MN, mamillary nucleus; Hi(gd), hippocampus (gyrus dentatus); SC, superior colliculus; LPTN, lateral posterior thalamic nucleus; LGB, lateral geniculate body; Hi, hippocampus; Cb, cerebellum; ICo, inferior colliculus; LC, locus coeruleus; DPN, dorsal parabrachial nucleus.

Table 1. Regional distribution of CRF receptors in cynomolgus monkey brain

Region	[[^{125}I]Tyr]oCRF binding, optical density × 10^3
Cerebral cortex	
Frontal	458 ± 2.7
Orbital	815 ± 8.9
Parietal	417 ± 4.0
Temporal	489 ± 3.4
Anterior cingulate	315 ± 4.4
Hippocampus	445 ± 3.7
Dentate gyrus	479 ± 6.2
Basal telencephalon	
Olfactory tubercle	305 ± 2.8
Lateral septal nuclei	152 ± 3.0
Septal area	131 ± 2.7
Nucleus accumbens	241 ± 2.6
Caudate	198 ± 3.2
Putamen	240 ± 3.8
Amygdaloid complex	376 ± 4.7
Bed nucleus stria terminalis	184 ± 4.8
Claustrum	325 ± 3.6
Cerebellum	
Granular layer	415 ± 3.0
Diencephalon	
Paraventricular thalamic nucleus	206 ± 5.8
Dorsomedial thalamic nucleus	289 ± 6.0
Anterior ventricular thalamic nucleus	158 ± 2.9
Posterior lateral thalamic nucleus	228 ± 5.0
Nucleus tuberalis lateralis	491 ± 4.9
Preoptic area	160 ± 3.3
Arcuate nucleus	620 ± 12
Mamillary bodies	381 ± 3.8
Lateral geniculate nucleus	184 ± 2.5
Brainstem	
Superior colliculus	198 ± 3.9
Inferior colliculus	235 ± 3.0
Locus coeruleus	110 ± 1.9
Substantia nigra	105 ± 2.2
Dorsal parabrachial nucleus	228 ± 2.4

Data are means ± SD of optical density values in three sections from one experiment after subtracting nonspecific background (260 ± 1.6). In each section the nonspecific background was uniform.

well developed only in primates, and especially in man. These areas receive abundant innervation from the dorsomedial nucleus of the thalamus, which relays impulses from several autonomic centers (15). This area of the thalamus, which plays an important role in emotional responses, also contains abundant CRF receptors.

CRF receptors were also found in two hypothalamic areas involved in the control of gonadotropin secretion, the preoptic area and the arcuate nucleus. A possible role for CRF in the control of sexual function has been supported by studies showing decreases in luteinizing hormone release (16) and inhibition of sexual behavior in the female rat (17) after CRF injection in the arcuate-ventromedial hypothalamic regions. The presence of CRF receptors in these areas in the primate brain suggests that CRF may also influence gonadotropin secretion in man and could be involved in the mechanism of altered gonadal function during prolonged stress. The arcuate nucleus has projections to a number of hypothalamic and limbic structures that are rich in CRF and also in ACTH and β-endorphin (18). Coexistence of CRF and opiocortin peptides has been described in many other structures that contain CRF receptors, such as the nucleus accumbens, stria terminalis, preoptic area, amygdala, geniculate bodies, locus coeruleus, and parabrachial nucleus (18). The similar anatomical distribution of CRF and its receptors and of opiocortin peptides suggests that, as in the pituitary gland, both systems may be functionally related in the brain.

Also in the hypothalamus, it is interesting to note the very high concentration of CRF receptors in the nucleus tuberis lateralis, a structure of yet unknown function. This nucleus is particularly developed in primates (19), and the abundance of CRF receptor at this site may provide some basis for the study of its function.

A high density of CRF receptors was also observed in the amygdala. This important component of the limbic system has both efferent and afferent connections with the cortical areas that contain the highest CRF receptor concentrations, namely, the frontal, orbital, cingulate, temporal, and insular cortices (20–22). The amygdaloid nuclei also receive projections from the locus coeruleus, hypothalamus, and dorsomedial thalamic nucleus and have efferent projections to the dorsomedial thalamic nucleus, nucleus stria terminalis, preoptic area, septal regions, and arcuate nucleus (19, 23–25). All of these areas that are connected to the amygdala were found to contain CRF receptors. It should be noted that the connections of the amygdala to the septal and preoptic area are unique for the higher mammals (24, 25), a feature that correlates with the presence of CRF receptors in these regions in the monkey but not in the rat (8). Electrical stimulation of the amygdala in experimental animals has been shown to cause arousal, attention, fear, and rage reactions associated with sympathetic activation (26–29). These reactions are similar to those observed during stress and after intracerebroventricular administration of CRF (1). Since CRF and its receptors are present in the amygdala, it is likely that the peptide may have a role in the generation of some of these responses.

High CRF receptor concentrations were also present in the limbic lobe, which is composed of the cingulate and parahippocampal cortex and the hippocampus. This structure, referred to by some authors as the "visceral brain" because of its close relationship with the hypothalamus, also has connections with other limbic structures and the neocortex (30). The limbic system has a primary role in the mechanisms that control behavior, emotion, and autonomic and endocrine function. A number of these limbic system-mediated responses can be mimicked by central administration of CRF. Intracerebroventricular injection of CRF in rat, dog, and monkey results in behavioral changes and activation of the hypothalamic-pituitary-adrenal axis (1, 4, 31, 32) and the sympathetic nervous system with the subsequent visceral and metabolic responses (1, 32–35). In chair-restrained monkeys, administration of CRF into the brain causes an increase in arousal consistent with limbic activation (31). In monkeys tested in a less restrictive setting, freely moving in their cages, large doses of CRF injected into the brain (180 μg) caused a depressed state with a combination of huddling and lying down behavior, similar to that observed during isolation stress in monkeys. This supports the hypothesis that excessive CRF activity in the central nervous system may be involved in certain types of depression in humans. Many patients with depression show hyperactivity of the pituitary-adrenal axis (36), with reduced ACTH responses to exogenous CRF, suggestive of increased endogenous CRF production (37). The presence of CRF receptors in the cerebral cortex and limbic system provides a mechanism through which increased activity of the CRF neurons could be expressed as the behavioral and visceral components of depression.

The involvement of CRF in control of the autonomic nervous system has been emphasized recently by studies demonstrating the presence of immunoreactive CRF (38, 39) and functional CRF receptors in the peripheral sympathetic nervous system. Recent studies have demonstrated receptors for CRF in the adrenal medulla in the rat (40) and in the

adrenal medulla and sympathetic ganglia in the monkey (41). Adrenal medullary receptors are also coupled to adenylate cyclase and their prolonged activation by CRF in cultured chromaffin cells results in increases in catecholamine and [Met]enkephalin release (41).

The presence of a peptide in nerve terminals at its sites of action in the brain is a criterion for consideration as a neurotransmitter. In the rat, extensive studies have demonstrated the presence of immunoreactive CRF in several extrahypothalamic areas including the sites at which CRF receptors are present. However, little information is available in this regard for monkey or man, for which studies have been limited mainly to descriptions of the hypothalamic pathways (42, 43). In the squirrel monkey, cell bodies and fibers with projections to the median eminence have been localized in the paraventricular and supraoptic nuclei of the hypothalamus (42). Similar localization of immunoreactive CRF to the paraventricular nucleus and median eminence has been described in the hypothalamus from human fetuses and newborns (43). No detectable CRF receptors were found in these hypothalamic areas. This finding, which agrees with previous observations in the rat (8), is consistent with the role of these areas as the source of CRF released to the portal circulation and not as targets for CRF action.

With respect to extrahypothalamic localization of the peptide, the presence of immunoreactive CRF has been observed in the circumventricular organs in *Macaca fuscata* brain (44), but there are no reports on CRF localization in the limbic system. Further studies are needed to clarify the extrahypothalamic localization of CRF in the primate brain.

Although the mechanisms by which CRF modulates neuronal activity and the exact physiological role of the peptide in the nervous system will require further study, the presence of functional CRF receptors in discrete structures in the brain suggests that CRF modulates central nervous system function. These findings support the view that CRF participates in the integrated behavioral, visceral, and endocrine responses to stress by acting through its specific receptors in the central and peripheral nervous system, as well as in the pituitary gland.

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