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CORTICOTROPIN-RELEASING FACTOR: PHYSIOLOGY, PHARMACOLOGY, AND ROLE IN CENTRAL NERVOUS SYSTEM DISORDERS

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HISTORICAL PERSPECTIVES

In 1948 Sir Geoffrey Harris first proposed the concept that the hypothalamus plays a primary role in the regulation of the pituitary-adrenocortical axis. Subsequently, during the 1950s, Guillemin and Rosenberg, and Saffran and Schally independently observed the presence of a factor in hypothalamic extracts (termed corticotropin-releasing factor, CRF) that could stimulate the release of adrenocorticotropic hormone (ACTH, corticotropin) from anterior pituitary cells *in vitro.* Although CRF was the first hypothalamic hypophysiotropic factor to be recognized, its chemical identity remained elusive until 1981, when Wylie Vale and colleagues at the Salk Institute reported the isolation, characterization, synthesis, and *in vitro* and *in vivo* biological activities of a 41-amino acid hypothalamic ovine CRF (1). Just over a decade later, Vale and colleagues were the first to report the cloning of the human CRF_1 receptor from a single human Cushing's corticotropic adenoma using an expression cloning techniques (2). This initial discovery led to the identification of a second receptor subtype (termed CRF_2), which has now been localized and characterized in a variety of species (see the following).

This chapter provides an overview of the CRF system and its related receptor targets. More detailed and comprehensive information on CRF is available in recent reviews (3,4) and books (5,6) on the topic.

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CHARACTERISTICS OF THE CRF PEPTIDE AND GENE SEQUENCES

Amino Acid Sequence and Structure of CRF

The sequence of CRF has been determined in a variety of species including sheep, humans, rats, pigs, goats, and cows. In all species, CRF is a 41-amino acid residue single chain polypeptide (Fig. 7.1). Rat and human CRFs are identical to one another and differ from ovine CRF by seven amino acid residues. All three CRFs have close amino acid homology and share some biological properties with sauvagine, a 40-amino acid peptide that exists in frog skin, and urotensin I, a 41-amino acid peptide derived from fish urophysis. Caprine and ovine CRF are identical and differ from bovine CRF by one amino acid. Porcine CRF more closely resembles rat and human CRF. CRF and related peptides are amidated at their carboxy terminal; CRF COOH-terminal-free acid has less than 0.1% of the potency of native CRF, suggesting the importance of amidation to biological activity of the peptide. Studies to determine the solution structure of CRF using proton nuclear magnetic resonance suggest that human CRF comprises an extended N-terminal tetrapeptide connected to a well-defined α-helix between residues 6 and 36 (7). An α-helical oCRF(9-41) has been demonstrated to be an antagonist of CRF (8), which underscores the necessity of the α-helical conformation for receptor binding and biological activity.

Organization of the CRF Gene and Protein Precursor

The nucleotide sequences encoding ovine and rat CRF cDNA precursors as well as human, rat, and ovine CRF

FIGURE 7.1. Amino acid sequences of ovine and rat/human corticotropin-releasing factor (CRF) and related peptides including human and rat urocortin. Rat CRF and human CRF are identical and differ from ovine CRF by seven amino acid residues, which are denoted by the shading. All peptides are amidated at the carboxy terminus; the amidation is essential for biological activity. See color version of figure.

genes have been determined (9,10). The locus of the CRF gene is on chromosome 8q13 in the human. The CRF genes are quite similar to one another, containing two exons separated by intervening intron 686 to 800 base pairs long. The first exon encodes most of the 5′-untranslated region of the mRNA and the second encodes the entire prepro-CRF precursor polypeptide, which is 187 to 196 amino acids long; the carboxy end of the precursor contains the 41-amino acid peptide sequence. The high incidence of homology among species suggests that the gene has been highly conserved through evolution.

As previously demonstrated for other systems, the 5′-flanking DNA sequences are most likely to contain the DNA sequence elements responsible for glucocorticoid, cAMP and phorbol ester regulation, tissue-specific expression, and enhancer activity. Although a consensus cAMP response element has been identified, located 200 base pairs upstream from the major transcription initiation site, no obvious glucocorticoid response elements or activation protein (AP) 1-binding elements are present. A potential AP-2 binding site, which may mediate the responses to protein kinase A and C, is present 150 base pairs upstream from the major start site.

ANATOMY OF CRF

Distribution of CRF in the CNS

The distribution and localization of CRF mRNA in the central nervous system (CNS) have been evaluated using Northern blot analysis and *in situ* hybridization histochemistry, respectively. Radioimmunoassay and immunohistochemical studies have been critical in the determination of the neuroanatomic organization of CRF immunoreactive cells and fibers in the CNS. Overall, there is good concordance between studies demonstrating a widespread distribution of CRF cell bodies and fibers in the CNS. Detailed descriptions of the organization of CRF immunoreactive cells and fibers in rat brain have been published (11–13).

Morphologic data clearly indicate that the paraventricular nucleus of the hypothalamus (PVN) is the major site of CRF-containing cell bodies that influence anterior pituitary hormone secretion. These neurons originate in the parvocellular portion of the PVN and send axon terminals to the capillaries of the median eminence. CRF is also present in a small group of PVN neurons that project to the lower brainstem and spinal cord; this group of neurons may be involved in regulating autonomic nervous system function. Other hypothalamic nuclei that contain CRF cell bodies include the medial preoptic area, dorsomedial nucleus, arcuate nucleus, posterior hypothalamus, and mammillary nuclei.

The neocortex contains primarily CRF interneurons with bipolar, vertically oriented cell bodies predominantly localized to the second and third layers of the cortex and projections to layers I and IV. In addition, scattered cells are present in the deeper layers that appear to be pyramidal cells. Although CRF-containing neurons are found throughout the neocortex, they are found in higher densities in the prefrontal, insular, and cingulate areas. CRF neurons in the cerebral cortex appear to be important in several behavioral actions of the peptide, including effects on cognitive processing; furthermore, dysfunction of these neurons may contribute to many CNS disorders (see the following).

Large and discrete populations of CRF perikarya are present in the central nucleus of the amygdala, bed nucleus of the stria terminalis, and substantia innominata. CRF neurons in the central nucleus of the amygdala project to the parvocellular regions of the PVN, the parabrachial nucleus of the brainstem, and thus may influence both neuroendocrine and autonomic function in addition to behavioral activity. CRF neurons originating in the bed nucleus of the stria terminalis send terminals to brainstem areas such as the parabrachial nuclei and dorsal vagal complex, which coordinate autonomic activity. CRF fibers also interconnect the amygdala with the bed nucleus of the stria terminalis and hypothalamus. Scattered CRF cells with a few fibers are also present in telencephalic areas such as regions of the amygdala in addition to the central nucleus, septum, diagonal band of Broca, olfactory bulb, and all aspects of hippocampal formation, including the pyramidal cells, dentate gyrus, and subiculum.

Several groups of CRF cell bodies are present throughout the brainstem. In the midbrain, CRF perikarya are present in the periaqueductal gray, Edinger-Westphal nucleus, dorsal raphe nucleus, and ventral tegmental nucleus. Projections from the dorsal-lateral tegmental nucleus to a variety of anterior brain areas such as the medial frontal cortex, septum, and thalamus have also been described. In the pons, CRF cell bodies are localized in the locus ceruleus, parabrachial nucleus, medial vestibular nucleus, paragigantocel-

lular nucleus, and periaqueductal gray. CRF neurons originating in the parabrachial nucleus project to the medial preoptic nucleus of the hypothalamus. In the medulla, the large groups of cell bodies are present in the nucleus of the solitary tract and dorsal vagal complex with ascending projections to the parabrachial nucleus. Scattered groups of cell bodies are also present in the medullary reticular formation, spinal trigeminal nucleus, external cuneate nucleus, and inferior olive. The inferior olive gives rise to a well-defined olivocerebellar CRF pathway with projections to the Purkinje cells of the cerebellum. No CRF cell bodies are present in the cerebellar formation.

Within the spinal cord, CRF cell bodies are present in laminae V to VII and X and in the intermediolateral column of the thoracic and lumbar cord. CRF fibers originating in the spinal cord form an ascending system terminating in the reticular formation, vestibular complex, central gray, and thalamus. This ascending CRF system may play an important role in modulating sensory input. In addition, spinal cord CRF neurons may represent preganglionic neurons that modulate sympathetic outflow.

Distribution of CRF in Peripheral Tissues

In addition to its CNS distribution, CRF has been localized in a variety of peripheral tissues (14). CRF-like immunoreactive fibers are present in the intermediate lobe of the pituitary; these fibers originate in the hypothalamus. A physiologic role has been proposed for CRF in regulating proopiomelanocortin (POMC)-derived peptide secretion from the intermediate pituitary. CRF has also been localized in the adrenal medulla of a variety of species and is increased following stimulation of the splanchnic nerve stimulation and hemorrhagic stress. CRF-like immunoreactivity and CRF mRNA have been detected in lymphocytes, where they may play a role in regulating immune function. Other tissues in which CRF has been localized include the testis (Leydig cells and advanced germ cells), pancreas, stomach, and small intestine. Although CRF is not detected in the circulation under normal circumstances, very high levels have been measured in the plasma of pregnant women; the source of CRF in pregnancy appears to be the placenta. (See CRF-Binding Protein.)

UROCORTIN: A NOVEL MAMMALIAN CRF-RELATED PEPTIDE

Urocortin is the newest member of the CRF peptide family and has been demonstrated to possess many of the intrinsic properties of CRF itself as well as some unique properties of its own. Originally, the nonmammalian CRF-related analogues urotensin I (teleost fish) (15) and sauvagine (frog) (16) were thought to subserve the functions of CRF in the respective species; however, the discovery of peptides even closer to the structure of CRF in those species (17,18) led to the suggestion that other forms of CRF may exist in mammals. Furthermore, with the cloning of the CRF_2 receptor subtype, it became apparent that because sauvagine and urotensin had even higher affinity and activity at this subtype than CRF itself (19), a mammalian form of these peptides may exist that would serve as the endogenous ligand for the CRF_2 subtype. Indeed, a mammalian form of urotensin was recently discovered termed ''urocortin'' and the cDNA cloned from the rat (20) and human (21), respectively.

Amino Acid Sequence and Structure of Urocortin

The sequence of urocortin has been determined in both rats and humans. In rat, urocortin was identified using a library derived from rat midbrain and a carp urotensin cDNA probe. A full-length cDNA was described and encoded a putative 40-amino acid peptide that was related to CRF (20). The human form was subsequently identified using a cDNA probe encoding the peptide region of rat urocortin and screening a human genomic library (21). The resulting putative peptide demonstrated 88% identity to rat at the nucleotide level and 95% identity at the amino acid level (Fig. 7.1). In both species, urocortin is a 40-amino acid residue single-chain polypeptide; the two forms differ by only two amino acids at positions 2 (Asn to Asp) and 4 (Ser to Pro) (21). In addition to the homology between the species, the deduced amino acid sequence of rat and human urocortin exhibits sequence identity with urotensin I (63%) and human CRF (45%) (20). Consistent with the other CRF-related peptides, urocortin is also amidated at its carboxy terminal, again suggesting the importance of amidation to this family of peptides (Fig. 7.1).

Anatomic Distribution of Urocortin

The distribution of urocortin in rat tissues was elucidated first by examining the cellular localization of urotensin-like immunoreactivity and correlating that distribution with the *in situ* hybridization of urocortin mRNA. The highest areas of correlation and overlapping localization were the Edinger-Westphal nucleus and the lateral superior olive (20). In addition, cellular staining was observed in the external plexiform layer of the rat olfactory bulb and lateral hypothalamus. Interestingly, terminal projection fields in the lateral septum also demonstrated distinct localization where CRF_2 receptors have been uniquely localized (see the following). Although the localization of urocortin appears to be in very discrete brain regions, these regions demonstrate no CRF mRNA, suggesting that urocortin subserves some unique functionality within the CRF system. As described in the following, its affinity and functional activity at the

CRF_2 receptor subtype suggest that this may be one endogenous ligand for this subtype.

In Vitro and *In Vivo* Pharmacologic Effects

Urocortin binds with high affinity to all the known effectors of CRF function, including CRF_1, $CRF_{2\alpha}$, CRF_{2B} receptors, and CRF-binding protein (described in the sections that follow). This profile makes urocortin unique in the CRF system because endogenous r/hCRF has been shown to have relatively low affinity for the CRF_2 receptor subtypes and oCRF, which also has lower affinity for the CRF_2 subtype, also has very low affinity at the CRF-binding protein. Urocortin binds to cells stably transfected with the CRF_1, $CRF_{2\alpha}$, or CRF_{2B} receptors with affinities in the 100 to 500 pM range and has 100 pM affinity for the CRF-BP (20). In *in vitro* studies, urocortin stimulates cAMP accumulation in cells transfected with either CRF receptor subtype, and is extremely potent in stimulating ACTH release from cultured anterior pituitary cells (20). The effects on the CRF_1 receptor subtype are comparable to the effects of CRF itself; however, the activities observed at both CRF_2 receptor isoforms are approximately tenfold more potent than CRF itself (20,21). Furthermore, as has been shown for CRF, the presence of CRF-BP can decrease the ability of urocortin to stimulate ACTH release *in vitro.* Moreover, specific CRF-BP inhibitors such as r/hCRF(9-33) can restore the ability of urocortin to stimulate ACTH, further confirming the functional activity of urocortin at the CRF-BP (21).

In unanesthetized freely moving rats, urocortin administered IV was fivefold more potent than CRF in increasing plasma ACTH concentrations and demonstrated a longer duration of action. Similarly, urocortin reduced mean arterial pressure more potently and for a longer period of time than CRF or urotensin I (20). Thus, although capable of interacting with the CRF_1 receptor with equivalent potency and activity, the anatomic distribution, localization, and potency at the CRF_2 subtypes support the notion that urocortin is likely one endogenous ligand for this receptor subtype. Clearly, further study is required to determine the specific role that this novel endogenous peptide plays in the regulation of the CRF system.

CRF RECEPTORS

Cloning of CRF Receptor Subtypes

Molecular cloning studies have enabled the elucidation of receptor subtypes for the CRF system. Structurally, the CRF receptor subtypes all contain seven putative transmembrane domains and share considerable sequence homology with one another. These receptors are members of the family of ''brain-gut'' neuropeptide receptors, which includes receptors for calcitonin, vasoactive intestinal peptide, parathyroid hormone, pituitary adenylate cyclase-activating peptide, growth hormone-releasing factor, glucagon, and secretin. In addition, the known members of this neuropeptide receptor family also belong to the superfamily of G-protein coupled receptors; thus far, all have been shown to stimulate adenylate cyclase in response to their respective agonist activation.

The CRF_1 receptor was cloned first from a human Cushing's corticotropic adenoma using an expression cloning technique and characterized as a 415-amino acid protein with potential N-linked glycosylation sites, protein kinase C phosphorylation sites in the first and second intracellular loops and in the C-terminal tail, as well as casein kinase II and protein kinase A phosphorylation sites in the third intracellular loop (2). Independently, this receptor subtype was also identified in mouse (22) and rat (23,24). In all three species, CRF_1 receptor mRNAs encode proteins of 415 amino acids, which are 98% identical to one another. The potential N-linked glycosylation sites on the N-terminal extracellular domain are characteristic of most G-protein coupled receptors and confirm the glycosylation profiles determined by chemical affinity cross-linking studies (25). In those studies, although the molecular weights of the proteins labeled from brain or pituitary appeared different when labeled with [^{125}I]oCRF, the deglycosylation and peptide mapping studies suggested that the protein itself was identical and that the differences were owing to posttranslational modification (25). Indeed, the molecular weight predicted from the deglycosylated forms of the CRF_1 receptor was virtually identical to that obtained from the cloned amino acid sequence. These data taken together established the fact that the CRF_1 receptor subtype is the dominant form in both the brain and pituitary.

CRF_2 Receptors

Following the cloning of the CRF_1 subtype, two forms of a second family member were discovered in the rat and termed $CRF_{2\alpha}$ and CRF_{2B}. The rat $CRF_{2\alpha}$ receptor (19) is a 411-amino acid protein with approximately 71% identity to the CRF_1 receptor. The CRF_{2B} receptor has been cloned from both rat (19) and mouse (24,26), and is a 431-amino acid protein that differs from the $CRF_{2\alpha}$ subtype in that the first 34 amino acids in the N-terminal extracellular domain are replaced by 54 different amino acids. The genomic structure and corresponding cDNA of the human $CRF_{2\alpha}$ receptor subtype was cloned and characterized. The cDNA sequence in the protein-coding region had 94% identity with the previously reported rat $CRF_{2\alpha}$ receptor (27). In addition, the human $CRF_{2\alpha}$ receptor protein was found to be a 411-amino acid protein that had an overall 70% identity with the human CRF_1 receptor sequence (less in the N-terminal extracellular domain; 47%). In stably transfected cells, the human $CRF_{2\alpha}$ receptor had the same pharmacologic characteristics as those demonstrated for the

rat and increased intracellular cAMP levels in response to either sauvagine or CRF (see the following for details). Very recently, the human form of the $CRF_{2\beta}$ receptor was cloned from human amygdala and demonstrated 94% identity to human $CRF_{2\alpha}$ receptors at the protein level. Preliminary characterization of this novel human isoform indicated that this form also had higher affinity for sauvagine and urotensin than for r/hCRF (28). The $CRF_{2\gamma}$ receptor was the most recently identified isoform and has thus far only been found in human brain. This splice variant uses yet a different 5′ alternative exon for its amino terminus and replaces the first 34-amino acid sequence of the $CRF_{2\alpha}$ receptor with a unique 20 amino acid sequence. Thus, although the CRF_2 receptor exists as three isoforms, $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$, there are at present no known functional splice variants for the CRF_1 receptor. Figure 7.2 illustrates the differences among human $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$ in the N-terminal extracellular domain. Between the CRF_1 and CRF_2 receptors, there exist very large regions of amino acid identity, particularly between transmembrane domains five and six. This similarity strongly argues for conservation of biochemical function because this region is thought to be the primary site of G-protein coupling and signal transduction. All three CRF_2 receptor subtypes contain five potential N-glycosylation sites, which are analogous to those found on the CRF_1 receptor subtype. The genomic structure of the human CRF_2 receptor gene is similar to that of the mouse CRF_1 receptor described in the preceding and has 12 introns, the last ten of which interrupt the coding region in identical positions. These gene sequences, however, diverge significantly at the 5′ end, and the chromosomal mapping of the human CRF_2 gene has been localized to chromosome 7 p21-p15.

Pharmacologic Characteristics

The literature is replete with information on the pharmacologic and biochemical characterization of CRF receptors in a variety of tissues and animal species. (See refs. 29 and 30–33 for reviews.) The radioligand binding characteristics of CRF receptors that have been performed thus far in brain, endocrine, and immune tissues have used the available radioligands at the time, which were [^{125}I]-Tyr^0 oCRF, [^{125}I]-Tyr^0 r/hCRF, and [^{125}I]-Nle^{21}-Tyr^{32} r/hCRF. These ligands have all demonstrated high affinity for the CRF_1 receptor subtype and lower affinity for the CRF_2 subtypes (as described in the following). Thus, the discovery of the CRF_2 receptor subtype and its isoforms has not confused the earlier literature owing to the apparent "selectivity" of the r/hCRF and oCRF analogue radioligands for the CRF_1 receptor. Recently, [^{125}I]-Tyr^0 sauvagine, a novel radioligand for the CRF_2 receptor, has been described that binds to both receptor subtypes with equal affinity and has become a useful tool in the study of CRF_2 receptors (34).

CRF receptors fulfill all of the criteria for bona fide receptors. The kinetic and pharmacologic characteristics of CRF_1 receptors are comparable in brain, pituitary, and spleen. The binding of [^{125}I]oCRF in a variety of tissue homogenates as well as in CRF_1 receptor-expressing cell lines is dependent on time, temperature, and tissue concentration, and is saturable, reversible, and of high affinity with K_d values of 200 to 400 pM. The pharmacologic rank order profile of these receptors from various tissues has been compared using closely related analogues of CRF. Bioactive analogues of CRF have high affinity for [^{125}I]oCRF binding sites, whereas biologically inactive fragments of the peptide and unrelated peptides are all without inhibitory binding activity in brain, endocrine, and immune tissues.

CRF_1 receptors exhibit the typical properties of neurotransmitter receptor systems linked to the adenylate cyclase system through a guanine nucleotide binding protein. In *in vitro* radioligand binding studies, divalent cations (e.g., magnesium ions) have been shown to enhance agonist binding to receptors coupled to guanine nucleotide binding proteins by stabilizing the high-affinity form of the receptor–effector complex. In contrast, guanine nucleotides have the ability to selectively decrease the affinity of agonists for their receptors by promoting the dissociation of the agonist high-affinity form of the receptor. Consistent with CRF receptors being coupled to a guanine nucleotide regulatory protein, the binding of [^{125}I]oCRF to pituitary, brain, and spleen homogenates is reciprocally increased by divalent cations such as Mg^{2+} and decreased by guanine nucleotides. Furthermore, in expressed cell lines using a β-galactosidase reporter system, CRF and related analogues could stimulate the production of β-galactosidase in whole cells with the same pharmacologic rank order of potencies as those in a variety of tissues from different species (35).

The cloning of the CRF_2 receptor subtype gave the first indication that other family members of this receptor system exist and have unique properties that could subserve functions that were previously undefined. As mentioned, a fundamental element in the characterization of any receptor system is the availability of high-affinity and selective ligands that can be used to label the proteins in a reversible manner. The initial observations clearly demonstrated that the CRF_2 receptor subtype recognized the nonmammalian analogues of CRF with high affinity (similar in profile to the CRF_1 subtype) but unlike the CRF_1 receptor, had low affinity for the endogenous CRF ligands (r/hCRF and its analogues) (19). Thus, the available radioligands used in the initial studies of CRF receptors were not useful in providing information about this subtype.

The development of a high-affinity radioligand suitable for the characterization of the CRF_2 receptor subtype was recently described (34). Using one of the high-affinity nonmammalian analogues of CRF (sauvagine), a radiolabel was developed, and its binding specificity and selectivity determined. [^{125}I]Tyr^0-sauvagine was found to bind reversibly, saturably, and with high affinity to both the human CRF_1

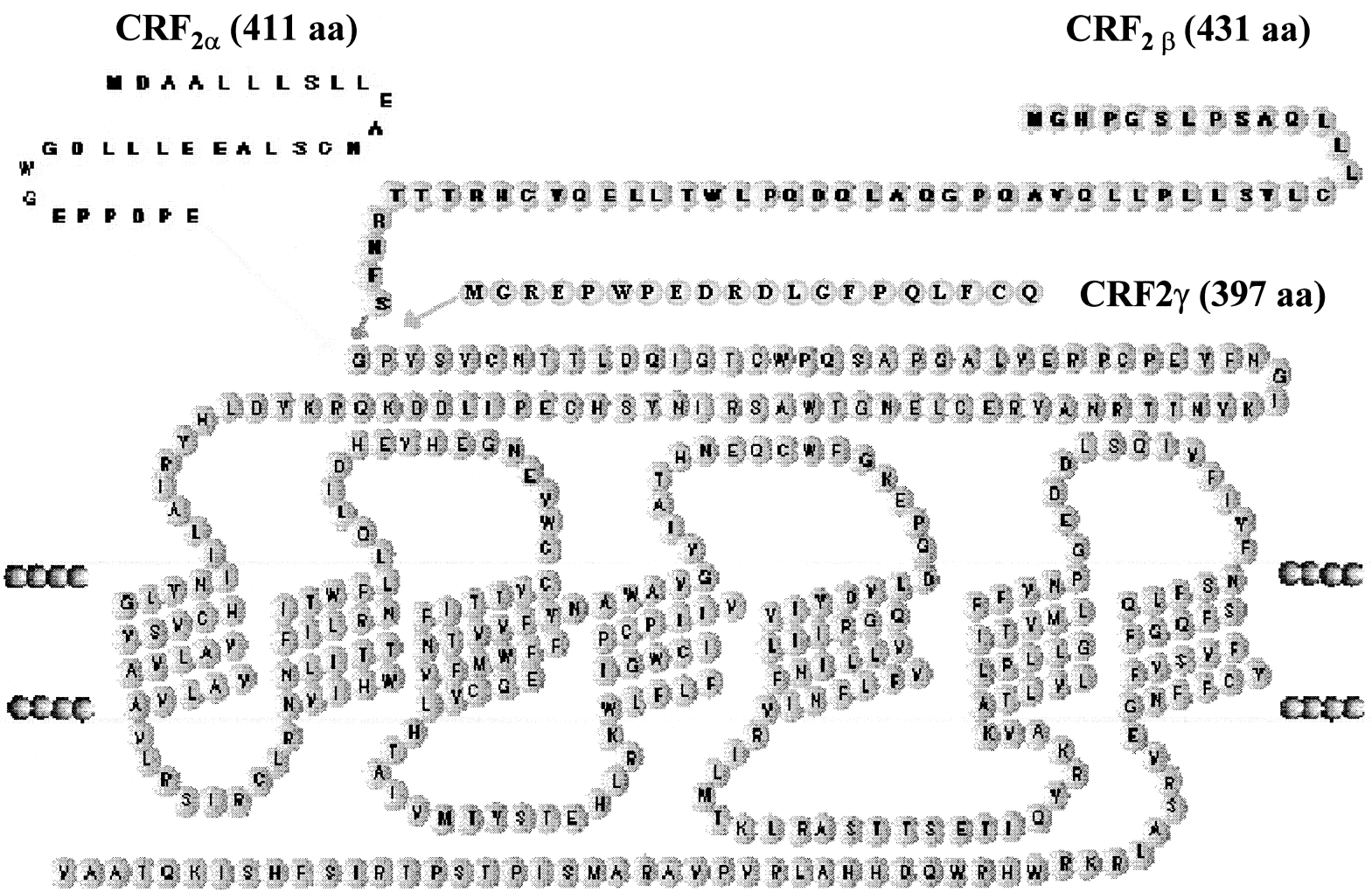

FIGURE 7.2. Amino acid sequences of the human $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$ receptor subtypes. The CRF_2 receptor isoforms are demonstrated with the seven putative transmembrane domains. The arrows indicate where the different CRF_2 receptor isoforms diverge at the N-terminus. See color version of figure.

and $CRF_{s\alpha}$ receptor subtypes expressed in mammalian cell lines. The specific signal for the labeling of the human $CRF_{a\alpha}$ receptors was greater than 85% over the entire concentration range of the radioligand, which suggested very low nonspecific binding in the expressed cell line. The radioligand bound in a reversible, time- and protein-dependent manner, reaching equilibrium within 60 minutes with the binding being stable for at least 4 hours at 22°C. Scatchard analyses demonstrated an affinity of about 200 pM for the CRF_2 receptor subtype and a maximum receptor density in the expressing cells of about 180 fmol/mg protein (34).

The pharmacologic rank order of potencies for the CRF_2 receptor labeled with [^{125}I]sauvagine was essentially identical to the *in vitro* effects of the same unlabeled peptides in the production of cAMP in cells expressing the receptor as described. That is, the nonmammalian analogues sauvagine and urotensin I that were more potent in stimulation of cAMP production were also more potent at inhibiting the binding of [^{125}I]sauvagine than oCRF or r/hCRF. Interestingly, the putative antagonists for CRF receptors, D-PheCRF(12-41) and α-helical CRF(9-41) exhibited approximately equal affinity for the two receptor subtypes either in inhibiting [^{125}I]sauvagine binding or inhibiting sauvagine-stimulated cAMP production (34). These data clearly indicated that although distinct pharmacologic differences exist between the two receptor subtypes of the same family (in terms of their rank order profile), they still must share some structural similarities. Further study is required to determine the precise common structural features of these two family members.

In addition to the pharmacologic rank order profile, [^{125}I]sauvagine binding to the CRF_2 receptor was guanine nucleotide-sensitive, confirming the agonist activity of this peptide for the receptor. Although there is as yet no direct evidence, this modulation of the binding of [^{125}I]sauvagine to the human $CRF_{2\alpha}$ receptor by guanine nucleotides suggests that this receptor exists in two affinity states for agonists coupled through a guanine nucleotide binding protein to its second messenger system. Unfortunately to date, the only ligands available for the biochemical study of these receptors have been agonists, making it very difficult to examine the proportions and affinities of high- and low-affinity states of these receptors. Further study is required, possibly using labeled antagonists as tools in order to characterize the affinity states of these receptors.

The high affinity of the nonmammalian CRF analogues for this subtype has raised the possibility that other endogenous mammalian ligands exist that have high affinity and selectivity for this receptor subtype. As described, the recent discovery of urocortin (36), although not selective for the CRF_2 subtype, has provided the first evidence for one such endogenous molecule that has high affinity for the CRF_2 receptor. With the increase in the complexity of the CRF system recently elucidated, it is highly likely that more members, from both the receptor and ligand families, remain to be discovered that will lead to a much more comprehensive understanding of this system and its role in both normal and pathologic physiology.

Autoradiographic Localization of CRF Receptor Subtypes

Many studies to date have described the distribution of CRF receptors in various tissues, including the pituitary, brain, and spleen (29,31–33). The autoradiographic localization of CRF_1 receptors in the anterior pituitary demonstrates a clustering of binding sites that corresponds to the distribution of corticotrophs. The intermediate lobe shows a more uniform distribution of binding sites characteristic of the homogeneous population of POMC-producing cells in this lobe. Overall, the distribution pattern of CRF_1 receptors within the pituitary supports the functional role of CRF as the primary physiological regulator of POMC-derived peptide secretion from the anterior and intermediate lobes of the pituitary.

Receptor autoradiography and binding studies in discrete areas of rat and primate CNS demonstrate that, in general, the highest concentration of CRF binding sites are distributed in brain regions involved in cognitive function (cerebral cortex), limbic areas involved in emotion and stress responses (amygdala, nucleus accumbens, and hippocampus), brainstem regions regulating autonomic function (locus ceruleus and nucleus of the solitary tract), and olfactory bulb. In addition, there is a high density of CRF_1 receptor sites in the molecular layer of the cerebellar cortex and the spinal cord where the highest concentrations are present in the dorsal horn.

CRF receptors in spleen are primarily localized to the red pulp and marginal zones. The localization of [^{125}I]oCRF binding sites in mouse spleen to regions known to have a high concentration of macrophages suggests that CRF receptors are present on resident splenic macrophages. The absence of specific [^{125}I]oCRF-binding sites in the periarteriole and peripheral follicular white pulp regions of the spleen suggests that neither T nor B lymphocytes have specific high-affinity CRF receptors comparable to those localized in the marginal zone and red pulp areas of the spleen or in the pituitary and brain.

The availability of nucleotide sequences for CRF_1 and CRF_2 receptors has allowed a detailed examination of the regional and cellular distribution of CRF receptor subtype mRNA expression utilizing both RNAse protection assays and *in situ* hybridization histochemistry. A comparison of the distribution of CRF_1 and CRF_2 mRNA and receptor protein defined by ligand autoradiography is demonstrated in adjacent horizontal sections of rat brain (Fig. 7.3).

The distribution of CRF_2 message clearly differs from that of the CRF_1 and exhibits a distinct subcortical pattern. In the rat brain, the CRF_1 mRNA was most abundant in neocortical, cerebellar, and sensory relay structures and gen-

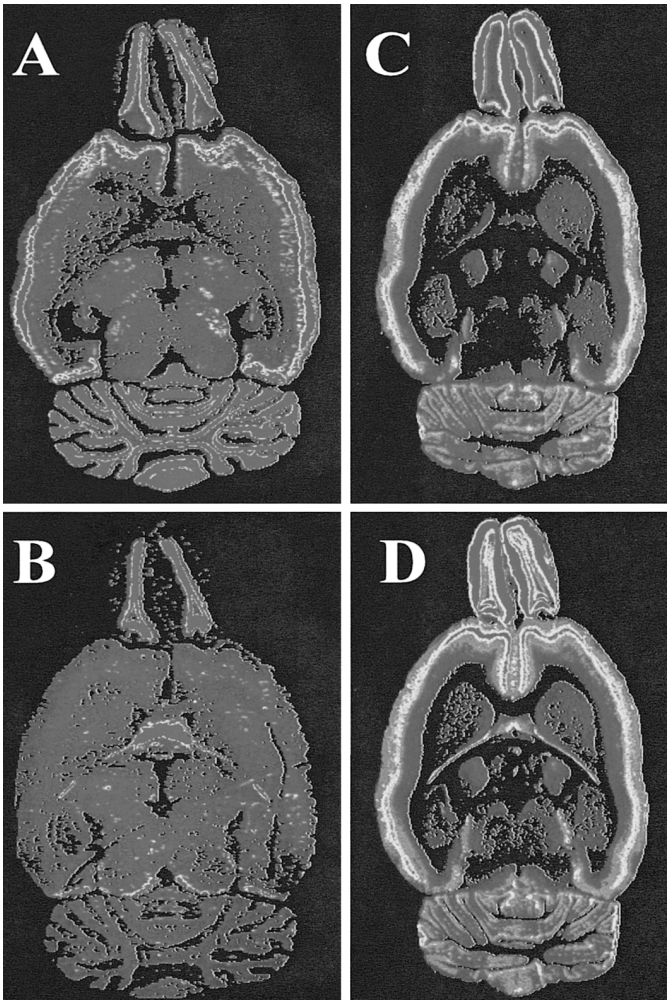

FIGURE 7.3. Digitized, color-coded images of CRF_1 *(Panel A)* and CRF_2 *(Panel B)* receptor mRNA expression and receptor autoradiography in adjacent horizontal sections of rat brain. The highest levels of mRNA expression are coded in red, whereas the lowest concentrations are coded in blue. Similarly, the highest densities of receptors labeled with either [^{125}I]oCRF (CRF_1 only; *Panel C*) or [^{125}I]sauvagine (CRF_1 and CRF_2; *Panel D*) are coded in red. There was a good correspondence between the message for a particular receptor subtype and its protein localization; the pharmacologic selectivity was retained for the two radioligands. (Compare *Panels C* and *D*.) See color version of figure.

erally corresponded to the previously reported distribution of [^{125}I]oCRF binding sites (Fig. 7.3). On the other hand, the CRF_2 receptor mRNA was localized primarily in subcortical regions such as the lateral septal nuclei, hypothalamic nuclei, bed nucleus of the stria terminalis, and amygdaloid nuclei. Using the radioligand [^{125}I]sauvagine described, CRF_2 receptors could be localized to areas of high CRF_2 message. In addition, because [^{125}I]sauvagine has equal affinity for both receptor subtypes (34), the autoradiography revealed the localization of both the CRF_1 and CRF_2 receptor subtypes, demonstrating the utility of this novel radioligand. (See ref. 37 for a complete and detailed account of the mRNA distribution patterns of CRF_1 and CRF_2 receptors.)

The heterogeneous distribution of CRF_1 and CRF_2 receptor mRNA and protein suggests distinctive functional roles for each receptor within the CRF system. For example, the lateral septum, by virtue of widespread reciprocal connections throughout the brain, is implicated in a variety of physiologic processes. These range from higher cognitive functions such as learning and memory to autonomic regulation, including food and water intake (38). In addition, the septum plays a central role in classical limbic circuitry and thus is important in a variety of emotional conditions, including fear and aggression. Thus, the lack of CRF_1 receptor expression in these nuclei suggests that CRF_2 receptors may solely mediate the postsynaptic actions of CRF inputs to this region and strongly suggests a role for CRF_2 receptors in modulating limbic circuitry at the level of septal activity. In addition, the selective expression of CRF_2 receptor mRNA within hypothalamic nuclei indicates that the anxiogenic and anorexic actions of CRF in these nuclei may likely be CRF_2 receptor-mediated. In contrast, within the pituitary, there is a predominance of CRF_1 receptor expression with little or no CRF_2 expression in either the intermediate and anterior lobes, indicating that it is the CRF_1 receptor that is primarily responsible for CRF regulation of the HPA axis.

In addition to the differences in distribution between the CRF_1 and CRF_2 receptor subtypes, there exists a distinct pattern of distribution between the CRF_2 isoforms ($CRF_{2\alpha}$ and CRF_{2B}) as well. The $CRF_{2\alpha}$ isoform is primarily expressed within the CNS, whereas the CRF_{2B} form is found both centrally and peripherally. Within the brain, the $CRF_{2\alpha}$ form is the predominant one, whereas the CRF_{2B} form is localized primarily to non-neuronal structures, the choroid plexus of the ventricular system, and cerebral arterioles (37,39). The identification of the CRF_{2B} form in cerebral arterioles suggests a mechanism through which CRF may directly modulate cerebral blood flow. Peripherally, the highest detectable levels of mRNA were found in heart and skeletal muscle with lower levels detected in lung and intestine (24,39). Taken together, the results of these studies demonstrating a distinct heterogeneous distribution pattern of CRF receptor subtypes in brain and peripheral tissues, strongly suggest that these receptor subtypes subserve very specific physiological roles in CRF related function both centrally and peripherally.

Second Messengers Coupled to CRF Receptors

Radioligand binding studies have demonstrated that CRF receptors in the brain-endocrine-immune axis are coupled to a guanine nucleotide regulatory protein. In all of these tissues, the primary second messenger system involved in transducing the actions of CRF is stimulation of cAMP

production (29,31–33,40). CRF initiates a cascade of enzymatic reactions in the pituitary gland beginning with the receptor-mediated stimulation of adenylate cyclase, which ultimately regulates POMC-peptide secretion and possibly synthesis. POMC-derived peptide secretion mediated by the activation of adenylate cyclase in the anterior and neurointermediate lobes of the pituitary is dose-related and exhibits appropriate pharmacology. Similarly in the brain and spleen, the pharmacologic rank order profile of CRF-related peptides for stimulation of adenylate cyclase is analogous to the profile seen in pituitary and in keeping with the affinities of these compounds for receptor binding. In addition, the putative CRF receptor antagonist α-hel ovine CRF(9-41) inhibits CRF-stimulated adenylate cyclase in brain and spleen homogenates.

In addition to the adenylate cyclase system, other signal transduction mechanisms may be involved in the actions of CRF. For example, CRF has been shown to increase protein carboxyl methylation, and phospholipid methylation in AtT-20 cells (41). Preliminary evidence suggests that CRF may regulate cellular responses through products of arachidonic acid metabolism (42). Furthermore, although the evidence in anterior pituitary cells suggests that CRF does not directly regulate phosphatidylinositol turnover or protein kinase C activity (42), stimulation of protein kinase C either directly or by specific ligands (vasopressin or angiotensin II), enhances CRF-stimulated adenylate cyclase activity, ACTH release, and inhibits phosphodiesterase activity (42). Thus, the effects of CRF on anterior pituitary cells and possibly in neurons and other cell types expressing CRF receptors are likely to involve complex interactions among several intracellular second messenger systems.

CRF-BINDING PROTEIN

CRF and Its Binding Protein in Human Plasma

Under normal conditions, the plasma levels of CRF remain low; however, CRF levels are markedly elevated in plasma during the late gestational stages of pregnancy (43–45). The source of the pregnancy-associated CRF is most likely the placenta because previous studies have demonstrated that the human placenta synthesizes CRF (46). The CRF in the maternal plasma is bioactive in releasing ACTH from cultured pituitary cells (44). In spite of the high levels of CRF in the maternal plasma, there is no evidence of markedly increased ACTH secretion or hypercortisolism in pregnant women (43). A plausible explanation for this paradoxic situation could be the presence of a binding protein in the plasma of pregnant women that could specifically inhibit the biological actions of CRF (44,45). This hypothesis was validated by the isolation of a CRF-binding protein (CRF-BP) from human plasma and its subsequent cloning and expression (see the following).

cDNA and Amino Acid Sequences

The CRF-BP was first isolated and purified to near homogeneity for sequencing and generation of oligonucleotide probes (47). Screening a human liver cDNA library using probes generated from the original amino acid sequence revealed a full-length cDNA containing a 1.8-kb insert that coded for a novel protein of 322 amino acids (48). A single putative N-linked glycosylation site was found at amino acid 203, which agrees with the previous observation of the presence of asparagine-linked sugar moieties on the native protein (49). Subsequent screening of a rat cerebral cortical cDNA library, revealed the presence of a single clone containing a 1.85-kb insert predicting a protein of 322 amino acids, which was 85% identical to the human CRF-BP. The putative glycosylation site on the rat protein seems to be conserved between the rat and human sequences (48). The pharmacology of these proteins appears to be similar with both the rat and human binding proteins having high affinity for the rat/human CRF ($K_d \sim 0.2$ nM) and very low affinity for the ovine form of CRF ($K_d \sim 250$ nM). Although there may be some similarities in the binding domains of the binding protein and the CRF receptor (as evidenced by the equal affinity of r/hCRF), these are distinct proteins, each with unique characteristics and distributions.

Distribution in Brain and Pituitary

Although the human and rat forms of the CRF-BP are homologous (as indicated), there is a somewhat different anatomic distribution pattern in the two species. The human form of the binding protein has been found abundantly in areas including liver, placenta, and brain, whereas in the rat levels of mRNA for the binding protein have only been localized in the brain and pituitary (48). Peripheral expression of the binding protein may have its greatest utility in the modulation and control of the elevated levels of CRF in circulating plasma induced by various normal physiologic conditions (see the preceding). In addition, expression of this binding protein in the brain and pituitary offers additional mechanisms by which CRF-related neuronal or neuroendocrine actions may be modulated.

CRF-binding protein has been localized to a variety of brain regions including neocortex, hippocampus (primarily in the dentate gyrus), and olfactory bulb. In the basal forebrain, mRNA is localized to the amygdaloid complex with a distinct lack of immunostained cells in the medial nucleus. CRF-binding protein immunoreactivity is also present in the brainstem particularly in the auditory, vestibular, and trigeminal systems, raphe nuclei of the midbrain and pons, and reticular formation (50). In addition, high expression levels of binding protein mRNA are seen in the anterior pituitary, predominantly restricted to the corticotrope cells. Expression of this protein in the corticotropes strongly suggests that the CRF-BP is involved in the regulation of neu-

roendocrine functions of CRF by limiting and/or affecting the interactions of CRF with its receptor, which is also known to reside on corticotropes; however, the detailed role of the binding protein in regulating pituitary–adrenal function remains to be elucidated.

CRF REGULATION OF NEUROENDOCRINE FUNCTION

Regulation of Pituitary Hormone Secretion

CRF is the major physiologic regulator of the basal and stress-induced release of ACTH, B-endorphin, and other POMC-derived peptides from the anterior pituitary. (See refs. 3, 4, and 51 for reviews.) CRF stimulates the release of POMC-derived peptides in anterior pituitary cells in culture and *in vivo;* these actions of CRF can be antagonized by the CRF receptor antagonist α-helical ovine CRF(9-41) or by immunoneutralization with an anti-CRF antibody. Several other lines of evidence support a critical role for endogenous CRF in regulating ACTH secretion. For example, increases in CRF in the hypophysial portal blood are observed following application of stress. Administration of CRF antisera or the CRF receptor antagonist results in attenuation of stress- or adrenalectomy-induced ACTH secretion further substantiating a role for CRF in regulating ACTH secretion from the anterior pituitary. In addition to effects in the anterior pituitary, CRF also has been reported to stimulate POMC-derived peptide secretion from the intermediate lobe of the pituitary gland.

Central administration of CRF inhibits the secretion of luteinizing hormone (LH) and growth hormone without any major effects on follicle-stimulating hormone, thyroid stimulating hormone, or prolactin secretion (3,4). The effects of CRF to inhibit LH secretion appear to be mediated at the hypothalamic level through effects of CRF to inhibit gonadotropin releasing hormone secretion. CRF-induced inhibition of LH secretion may also involve endogenous opioids since the effects are attenuated by administration of naloxone or antiserum to B-endorphin (3,4).

Regulation of Hypothalamic CRF Release

Plotsky and associates (52) and Owens and Nemeroff (4) provide a comprehensive review of the neurotransmitter regulation of hypothalamic CRF release. Most studies demonstrate stimulatory effects of cholinergic and serotonergic neurons on CRF release. The muscarinic and/or nicotinic cholinergic receptor subtypes involved in the stimulatory effects of acetylcholine on CRF secretion remain to be precisely elucidated. The effects of serotonin to stimulate CRF release appear to be mediated by a variety of receptor subtypes, including 5-HT_2, 5-HT_{1A}, and 5-HT_{1C} receptors. The effects of catecholamines and opioids on hypothalamic CRF release are less well defined. Norepinephrine has been reported to have both stimulatory and inhibitory effects on CRF release that may be a consequence of the dose administered as well as the receptor subtype involved. For example, in studies sampling hypophysial portal concentrations of CRF, Plotsky and colleagues (52) noted that low doses of norepinephrine stimulated CRF release *in vivo* via α-adrenergic receptors and inhibited CRF release at high doses via B-adrenergic receptors. Similarly, opioids have been reported to either inhibit or stimulate CRF release depending on the nature of the opioid tested, dose utilized, and receptor specificity (μ versus κ) involved. Drugs acting at GABA–benzodiazepine–chloride ionophore complex are potent inhibitors of CRF secretion.

Stress is a potent general activator of CRF release from the hypothalamus. The extent and time course of changes in CRF in the paraventricular nucleus and median eminence of the hypothalamus following application of stress are highly dependent on the nature of the stressor as well as the state of the animal. The effects of stress to increase the release and synthesis of CRF are mediated by many of neurotransmitter systems described in the preceding.

Glucocorticoids, which are involved in the negative feedback regulation the hypothalamic–pituitary–adrenocortical axis, are potent inhibitors of CRF release. Conversely, the absence of glucocorticoids following adrenalectomy results in marked elevations in the synthesis and release of CRF. The actions of glucocorticoids to inhibit CRF release are mediated directly at the level of the paraventricular nucleus of the hypothalamus as well as indirectly through actions on receptors in the hippocampus.

Modulation of Pituitary CRF Receptors

Stress (29,32,33,53) or adrenalectomy (29,32,33) result in hypersecretion of CRF and a consequent down-regulation of receptors in the anterior pituitary. The adrenalectomy-induced decreases in anterior pituitary receptors can be prevented by glucocorticoid replacement with corticosterone or dexamethasone (29,32,33). In addition, chronic administration of corticosterone has been reported to cause dose-dependent decreases in anterior pituitary CRF receptor number (29,32,33). An age-related decline in anterior pituitary CRF receptors has also been reported (54). In contrast, lesions of the paraventricular nucleus that result in dramatic reductions in hypothalamic CRF secretion have been reported to increase the density of pituitary CRF receptors (4). Thus, CRF receptors in the anterior pituitary appear to be reciprocally regulated by hypothalamic CRF release.

CRF REGULATION OF CNS ACTIVITY

Electrophysiologic Effects of CRF

CRF stimulates the electrical activity of neurons in various brain regions that contain CRF and CRF receptors, includ-

ing locus ceruleus (55), hippocampus (56), cerebral cortex, and hypothalamus as well as in lumbar spinal cord motor neurons (3,4). In contrast, CRF has inhibitory actions in the lateral septum, thalamus, and hypothalamic PVN (3, 4). The electrophysiologic effects of CRF on spontaneous and sensory-evoked activity of locus ceruleus neurons are well documented (55). Activation of the locus ceruleus, a brainstem nucleus comprising of noradrenergic cells, results in arousal and increased vigilance. Furthermore, dysfunction of this nucleus has been implicated in the pathophysiology of depression and anxiety. Centrally administered CRF increases the spontaneous discharge rate of the locus ceruleus in both anesthetized and unanesthetized rats, while decreasing evoked activity in the nucleus (55). Thus, the overall effect of CRF in the locus ceruleus is to decrease the signal to noise ratio between evoked and spontaneous discharge rates.

The effects of CRF on EEG activity have been reviewed in detail (3,4,57). CRF causes a generalized increase in EEG activity associated with increased vigilance and decreased sleep time. At CRF doses below those affecting locomotor activity or pituitary–adrenal function, rats remain awake, vigilant and display decreases in slow-wave sleep compared to saline-injected controls (57). Higher doses of the peptide, on the other hand, cause seizure activity that is indistinguishable from seizures produced by electrical kindling of the amygdala, further confirming the role of CRF in brain activation.

Autonomic Effects of CRF

A great deal of anatomic, pharmacologic, and physiologic data support the concept that CRF acts within the CNS to modulate the autonomic nervous system (3,4,58,59). For example, central administration of CRF results in activation of the sympathetic nervous system resulting in stimulation of epinephrine secretion from the adrenal medulla and noradrenergic outflow to the heart, kidney, and vascular beds. Other consequences of central administration of CRF include increases in the mean arterial pressure and heart rate. These cardiovascular effects of CRF can be blocked by the ganglionic blocker chlorisondamine underscoring the sympathetic actions of the peptide. In contrast, CRF acts in brain to inhibit cardiac parasympathetic nervous activity. (See ref. 58 for review.) Peripheral administration of CRF causes vasodilation and hypotension in a variety of species including humans (3,4,58,59). The physiologic role of CRF in regulating the autonomic nervous system is supported by data demonstrating central effects of the CRF receptor antagonist, α-helical ovine CRF(9-41) to attenuate adrenal epinephrine secretion resulting from stressors such as insulin-induced hypoglycemia, hemorrhage, and exposure to ether vapor (59). Overall, these data substantiate a major role for CRF in coordinating the autonomic responses to stress.

Gastrointestinal Effects of CRF

Studies examining the gastrointestinal effects of CRF have determined that CRF modulates gastrointestinal activity by acting at central and possibly peripheral sites, and that these effects are qualitatively similar to those observed following exposure to various stressors. (See refs. 3, 4, and 60 for reviews.) CRF inhibits gastric acid secretion, gastric emptying, and intestinal transit while stimulating colonic transit and fecal excretion in a dose-dependent manner when administered centrally or systemically to dogs or rats. CRF is equipotent in inhibiting gastric emptying in both species following both central and peripheral routes of administration. The central effects of CRF on gastric acid secretion do not appear to result from leakage of the peptide into peripheral blood because measurable quantities of CRF are not present in the circulation following injection of CRF into the third ventricle of the dog. Furthermore, an intravenous injection of anti-CRF serum completely abolishes the peripheral but not the central effect of CRF on gastric acid secretion. These data strongly implicate CRF in the mechanisms through which various stressors alter gastrointestinal function and are consistent with its proposed role in integrating the autonomic nervous system's response to stress.

Behavioral Effects of CRF

The behavioral effects of CRF in the CNS have been reviewed extensively (3,4,61). The effects of CRF on behavior are dependent on both the dose of peptide administered and the specific conditions under which the tests are performed. In a familiar or "home" environment, central administration of CRF produces a profound increase in locomotor activity. Although very low doses of CRF produce locomotor activation when tested in an open field test, higher doses produce a dramatic decrease in locomotor activity. CRF administered intracerebrally also produces additional behavioral effects including increases in sniffing, grooming, and rearing in a familiar environment, increased "emotionality" and assumption of a freeze posture in a foreign environment, decreased feeding and sexual behavior, and increased conflict behavior. The behavioral effects of CRF are not an indirect consequence of actions of the peptide to activate pituitary–adrenocortical hormone secretion because they are not seen following peripheral administration of CRF or following pretreatment with doses of dexamethasone that adequately block pituitary–adrenal activation. Of critical importance is the observation that these effects of CRF can all be blocked by administration of the peptide antagonist α-helical ovine CRF(9-41), strongly supporting a specific CRF receptor-mediated event in these behaviors. Furthermore, the CRF receptor antagonist by itself attenuates many of the behavioral consequences of stress underscoring the role of endogenous peptide in mediating many of the stress-related behaviors.

ROLE FOR CRF IN NEUROPSYCHIATRIC DISORDERS AND NEURODEGENERATIVE DISEASES

Major Depression and Anxiety Disorders

Many patients with major depression are hypercortisolemic and exhibit an abnormal dexamethasone suppression test. Given the primary role of CRF in stimulating pituitary–adrenocortical secretion, the hypothesis has been put forth that hypersecretion or hyperactivity of CRF in brain might underlie the hypercortisolemia and symptomatology seen in major depression. (See refs. 62 and 63 for review.) The concentration of CRF is significantly increased in the cerebrospinal fluid (CSF) of drug-free individuals (4,64,65), and a significant positive correlation is observed between CRF concentrations in the CSF and the degree of postdexamethasone suppression of plasma cortisol (66). Furthermore, the observation of a decrease in CRF binding sites in the frontal cerebral cortex of suicide victims compared to controls is consistent with the hypothesis that CRF is hypersecreted in major depression (67). The elevated CSF concentrations of CRF seen in depressed individuals are decreased following treatment with electroconvulsive therapy (68). In addition, a blunted ACTH response to intravenously administered ovine or human CRF is observed in depressed patients when compared to normal controls (69). The blunted ACTH response to exogenous CRF seen in depressed patients may be caused by the intact negative feedback of cortisol on the corticotrophs, a compensatory decrease in CRF receptors subsequent to chronic hypersecretion of the peptide, and/or desensitization of the pituitary corticotrophs to respond to CRF.

A number of studies suggest that anxiety-related disorders (e.g., panic disorder and generalized anxiety disorder) and depression are independent syndromes that share both clinical and biological characteristics. The role that has been proposed for CRF in major depressive disorders along with preclinical data in rats demonstrating effects of CRF administration to produce several behavioral effects characteristic of anxiogenic compounds (61) have led to the suggestion that CRF may also be involved in anxiety-related disorders. A role for CRF in panic disorder has been suggested by observations of blunted ACTH responses to intravenously administered CRF in panic disorder patients when compared to controls (70). The blunted ACTH response to CRF in panic disorder patients most likely reflects a process occurring at or above the hypothalamus, resulting in excess secretion of endogenous CRF.

Anorexia Nervosa

Anorexia nervosa is an eating disorder characterized by tremendous weight loss in the pursuit of thinness. There is similar pathophysiology in anorexia nervosa and depression, including the manifestation of hypercortisolism, hypothalamic hypogonadism, and anorexia. Furthermore, the incidence of depression in anorexia nervosa patients is high. Like depressed patients, anorexics show a markedly attenuated ACTH response to intravenously administered CRF (4,64,65). When the underweight anorexic subjects are studied after their body weight had been restored to normal, their basal hypercortisolism, increased levels of CRF in the CSF, and diminished ACTH response to exogenous CRF all return to normal at varying periods during the recovery phase (4,64,65). CRF can potently inhibit food consumption in rats, which further suggests that the hypersecretion of CRF may be responsible for the weight loss observed in anorexics. In addition, the observation that central administration of CRF diminishes a variety of reproductive functions (4,65) lends relevance to the clinical observations of hypogonadism in anorexics.

Alzheimer's Disease

Several studies have provided evidence in support of alterations in CRF in Alzheimer's disease (AD) (4,65,71–74). There are decreases in CRF content and reciprocal increases in CRF receptors in cerebral cortical areas affected in AD such as the temporal, parietal, and occipital cortex. The reductions in CRF and increases in CRF receptors are all greater than 50% of the corresponding control values. The up-regulation in cerebral cortical CRF receptors in AD under conditions in which the endogenous peptide is reduced suggests that CRF-receptive cells may be preserved in the cortex in AD. Chemical cross-linking studies have demonstrated a normal pattern of labeling of cerebral cortical CRF receptors in AD when compared to age-matched controls (75). Although these decreases in CRF content have a modulatory action on the receptors (up-regulation), there appears to be no effect on the concentration or levels of CRF-binding protein in cerebral cortical areas affected in AD (76). The reduction in cortical CRF content may be owing to selective degeneration of CRF neurons intrinsic to the cerebral cortex or dysfunction of CRF neurons innervating the cortex from other brain areas. Additional evidence for a role for CRF in AD is provided by observations of decreases in CRF in other brain areas including the caudate (71) and decreased concentrations of CRF in the CSF (77,78). Furthermore, a significant correlation is evident between CSF CRF and the global neuropsychological impairment ratings, suggesting that greater cognitive impairment is associated with lower CSF concentrations of CRF (79).

Immunocytochemical observations demonstrating morphologic alterations in CRF neurons in AD complement the studies described in the preceding. In AD, swollen, tortuous CRF-immunostained axons, termed fiber abnormalities, are clearly distinguishable from the surrounding normal neurons and are also seen in conjunction with amyloid deposits associated with senile plaques (80). Furthermore, the total

number of CRF-immunostained axons is reduced in the amygdala of Alzheimer's patients (80). Interestingly, the expression of CRF antigen in neurons is not globally reduced in Alzheimer's patients. CRF immunostaining of perikarya and axons located in the hypothalamic paraventricular nucleus is much more intense in AD patients than controls (80). Increased immunostaining of the paraventricular neurons in AD, if truly representative of increased content of CRF, could be related to increased amounts of CRF mRNA in these cells or increased translation of available mRNA. The increased expression and/or release of CRF from the paraventricular nucleus of the hypothalamus would provide a reasonable explanation for the hypercortisolemia often seen in Alzheimer's patients.

At present, the cerebral cortical cholinergic deficiency seems to be the most severe and consistent deficit associated with AD. Reductions in cerebral cortical CRF correlate with decreases in choline acetyl transferase (ChAT) activity (72). In Alzheimer's, there are significant positive correlations between ChAT activity and reduced CRF in the frontal, temporal, and occipital lobes. Similarly, significant negative correlations exist between decreased ChAT activity and increased number of CRF receptors in the three cortices. These data suggest that the reported reciprocal changes in presynaptic and postsynaptic markers in CRF in cerebral cortex of patients with AD may be, in part, a consequence of deficits in the cholinergic projections to the cerebral cortex. Additional studies are necessary to determine the functional significance of the interaction between CRF and cholinergic systems.

Other Neurological Disorders

Alternations in brain concentrations of CRF have been reported in other neurological diseases. For example, in cases of Parkinson's disease (PD) with dementia that also show pathologic features of AD, CRF content is decreased and shows a pattern similar to those cases exhibiting the pathology of AD alone (74,81). Specimens from patients with PD who did not have the histopathology characteristic of AD also demonstrate reductions of CRF content, although the reductions are less marked than in cases of combined AD and PD. Normal levels of CRF have been reported in the hypothalamus in PD (82), suggesting that the loss of CRF in the cerebral cortex is not generalized. CRF is deceased to approximately 50% of the control values in the frontal, temporal, and occipital lobes of patients with progressive supranuclear palsy (74,81), a rare neurodegenerative disorder that shares certain clinical and pathologic features with AD.

The similarity of the changes in CRF found in the context of the three neurological diseases associated with Alzheimer-type pathology raised the possibility that cerebral cortical reduction is nothing more than a nonspecific sequela of the disease process. In Huntington's disease (HD), a neurological disorder in which minimal cerebral cortical pathology is present, the CRF content in the frontal, temporal, parietal, occipital, and cingulate cortices and in the globus pallidus is not significantly different from that seen in neurologically normal controls (73). However, the CRF content in the caudate nucleus and putamen of the basal ganglia (a brain area that is severely affected in the disease) is less than 40% of the CRF concentrations seen in controls (73). The localization of the CRF changes to only affected brain regions in the four neurodegenerative disorders described suggest that CRF has an important role in the pathology of these dementias.

POTENTIAL THERAPEUTIC STRATEGIES DESIGNED FOR THE CRF SYSTEM

Nonpeptide CRF-Binding Protein Ligand Inhibitors

As mentioned, the CRF-binding protein has the capacity to bind and functionally inactivate CRF. Peptide CRF-BP ligand inhibitors have been shown *in vitro,* to release CRF from the binding protein making it available for binding to its receptor and have been theorized to have efficacy in diseases that are associated with low levels of CRF such as Alzheimer's disease (76). Interestingly, unlike the direct i.c.v. administration of CRF, inhibition of the CRF-BP by ligand inhibitors that release functional CRF does not cause anxiogenic-like activity in animal models, validating the approach for diseases that require an increase in CRF function (76). Thus, compounds that act to dissociate CRF from its binding protein complex will act to selectively increase synaptic concentrations of CRF in discrete brain regions and may provide a novel treatment opportunity for disorders associated with low levels of CRF. To date, however, no nonpeptide CRF-BP ligand inhibitors have been discovered to test this hypothesis. On the other hand, small molecule nonpeptide CRF receptor antagonists have shown encouraging preliminary results in this complex system.

Nonpeptide CRF Receptor Antagonists

As indicated in this and previous reviews of the CRF system, there are a number of neuropsychiatric indications where clinical and preclinical data have shown that the CRF system is hyperactivated as evidenced by abnormally high levels of CRF within the CNS. For example, in clinical studies of major depression, increased CRF concentrations in the cerebrospinal fluid, increased HPA activity, blunted ACTH responsiveness to exogenously administered CRF, and pituitary and adrenal hypertrophy have all indicated hypersecretion of CRF associated with this disorder. (See refs. 4, 5, 32, 65, and 83 for review.) It follows that for these indications the therapeutic strategy would involve functional blockade of the actions of CRF. This can be achieved

through inhibition of CRF synthesis and secretion, inactivation of CRF (either by antibody neutralization or increased metabolism), or direct antagonism by specific receptor blockade. Although anti-CRF antibodies have demonstrated antiinflammatory effects (84), these types of therapies are limited to peripheral use and could not be readily formulated for oral administration for central activity. Nevertheless, these therapeutics could be useful for the treatment of disorders with peripheral elevations of CRF such as rheumatoid arthritis.

The best strategy for the blockade of elevated CRF levels is to design specific and selective nonpeptide receptor antagonists. Particularly for use in the brain, these molecules can be designed to have receptor subtype specificity, good oral bioavailability, and rapid penetration across the blood–brain barrier; characteristics that are difficult to optimize with peptide therapies. The recent surge in combinatorial chemistry techniques, coupled with recent technological advances in robotic high-throughput screening and data management of large libraries of molecules have enabled the field of small molecule drug discovery. These advancements have led to the identification of several patented structural series of molecules known to antagonize the effects of CRF at the CRF_1 receptor subtype. (See ref. 85 for a complete review.) Several of these small molecules have recently appeared in the literature and reported to have good CRF_1 receptor antagonistic activity. Compounds such as CP 154,526 (86), NBI 27914 (87), Antalarmin (88), and most recently DMP 696 (89), all demonstrate a good *in vitro* profile, showing selectivity for the CRF_1 receptor subtype. Systemic administration of these compounds have been found to attenuate stress-induced elevations in plasma ACTH levels in rats, demonstrating that CRF_1 receptors can be blocked in the periphery. Furthermore, nonpeptide CRF_1 antagonists administered peripherally have also been demonstrated to inhibit CRF-induced seizure activity (90). Until recently, however, these compounds have suffered from poor solubility and pharmacokinetics, thus limiting their utility in *in vivo* characterization of the individual compounds as well as the overall proof of concept for the mechanism of CRF receptor efficacy. One compound has very recently been described as a water-soluble nonpeptide CRF_1 receptor antagonist (NBI 30775, also referred to as R121919) that demonstrates high affinity, and has a superior *in vitro* and *in vivo* profile compared to other nonpeptide CRF receptor antagonists (91).

NBI 30775, is a pyrazolopyrimidine with high affinity for the CRF_1 receptor and over 1,000-fold weaker activity at the CRF_2 receptor subtype. This compound does not interact at all with the CRF binding protein and was shown to be as potent as the peptide antagonist D-Phe CRF(12-41) at inhibiting the CRF-stimulated cAMP accumulation from cells that express the human CRF_1 receptor and CRF-stimulated ACTH release from cultured rat anterior pituitary cells *in vitro. In vivo,* this molecule potently attenuated the plasma elevations of ACTH observed following a stressful stimulus in the rat, and demonstrated both dose- and time-dependent CRF_1 receptor occupancy concomitant with the levels of drug measured in whole brain (91). Owing to the promising preclinical profile of this compound this particular compound was assessed in full Phase I clinical trials and an initial open label Phase IIa study where the compound was assessed in patients with major depressive disorder.

First Clinical Experience with CRF_1 Receptor Antagonists

Preclinically, studies have demonstrated that attenuation of the CRF system, either by decreasing synthesis and release or by selective blockade of the CRF receptor, results in decreased anxiety and behavioral activation in stressed animals; however, clinically it will probably not be beneficial to the overall outcome of the patient if the stress axis is maximally compromised. The preclinical studies described, prompted the development of NBI 30775 in Phase I safety studies in humans and in an open-label clinical trial in patients with major depressive disorder (92). In this latter study severely depressed patients were given NBI 30775 orally once daily in a dose-escalating manner and their hypothalamic-pituitary adrenal (HPA) function assessed. In addition, the patients' level of depression or anxiety was measured using the Hamilton depression (HAM-D) and anxiety (HAM-A) scales. The results demonstrated that this compound was safe and well tolerated under the conditions of this study. Moreover, the data suggested that blockade of the CRF_1 receptor in these patients did not result in an impairment of the HPA axis either at baseline or following an exogenous CRF challenge (92). This demonstration was critical in setting this potential therapy apart from existing therapies that blunt basal functioning of multiple neurotransmitter systems. Furthermore, although under the limited conditions of an open-label trial, there was a statistically significant dose-dependent reduction in the depression and anxiety scores using both clinician and patient ratings, suggesting that this mechanism may provide an exciting novel therapy in patients suffering with major depressive disorder. Although it is of great importance at this stage to develop these compounds as tools in the ultimate understanding of the CRF system and the role it plays in neuropsychiatric disorders, evidence is now beginning to emerge that compounds of this class, and more importantly of this mechanism, will prove beneficial in neuropsychiatric disorders such as depression or anxiety. Should compounds such as this continue to demonstrate efficacy in these disorders without severely compromising the stress axis as a whole, they would validate the CRF hypothesis for depression and anxiety and provide an entirely novel treatment for these devastating diseases.

CONCLUSION

Corticotropin-releasing factor is the key regulator of the organism's overall response to stress. CRF has hormone-like effects at the pituitary level to regulate ACTH secretion that, in turn coordinates the synthesis and secretion of glucocorticoids from the adrenal cortex. CRF also functions as a bona fide neurotransmitter in the CNS. CRF neurons and receptors are widely distributed in the CNS and play a critical role in coordinating the autonomic, electrophysiologic, and behavioral responses to stress.

Clinical data have implicated CRF in the etiology and pathophysiology of various endocrine, psychiatric, and neurological disorders. Hypersecretion of CRF in brain may contribute to the symptomatology seen in neuropsychiatric disorders such as depression, anxiety-related disorders, and anorexia nervosa. In contrast, deficits in brain CRF are apparent in neurodegenerative disorders such as AD, PD, and HD as they relate to dysfunction of CRF neurons in brain areas affected in the particular disorder. The recent discovery of novel receptor family members as well as novel alternative ligands for these subtypes serve not only to increase our understanding of the system but provide a basis for selective and rational drug design for the treatment of disorders that are associated with aberrant levels of CRF. Strategies directed at developing specific and selective CRF agents have yielded many nonpeptide small molecule CRF_1 receptor antagonists and a preliminary proof-of-concept has encouraged the further development of such agents. Compounds such as those described in this chapter may hold promise for novel therapies for the treatment of these various neuropsychiatric disorders without severely compromising this highly complex hormonal system. Clearly with the recent advances made within a very short period of time, it now seems possible to begin a full understanding of this increasingly complex neurohormone system.

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