Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary

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ABSTRACT   Corticotropin-releasing factor (CRF) is a major hypophysiotropic peptide regulating pituitary–adrenal response to stress, and it is also widely expressed in the central nervous system. The recent cloning of cDNAs encoding the human and rat CRF receptors has enabled us to map the distribution of cells expressing CRF receptor mRNA in rat brain and pituitary by in situ hybridization. Receptor expression in the forebrain is dominated by widespread signal throughout all areas of the neo-, olfactory, and hippocampal cortices. Other prominent sites of CRF receptor mRNA expression include subcortical limbic structures in the septal region and amygdala. In the diencephalon, low levels of expression are seen in a few discrete ventral thalamic and medial hypothalamic nuclei. CRF receptor expression in hypothalamic neurosecretory structures, including the paraventricular nucleus and median eminence, is generally low. In the brainstem, certain relay nuclei associated with the somatic (including trigeminal), auditory, vestibular, and visceral sensory systems, constituted prominent sites of CRF receptor mRNA expression. In addition, high levels of this transcript are present in the cerebellar cortex and deep nuclei, along with many precerebellar nuclei. In the pituitary, moderate levels of CRF receptor mRNA expression were detected throughout the intermediate lobe and in a subset of cells in the anterior lobe identified as corticotropes by concurrent immunolabeling. Overall, the central distribution of CRF receptor mRNA expression is similar to, though more expansive than, that of regions reported to bind CRF, and it shows limited overlap with loci expressing CRF-binding protein. Interestingly, CRF receptor mRNA is low or undetectable in several cell groups implicated as central sites of CRF action.

Corticotropin-releasing factor (CRF) is a 41-amino-acid neuropeptide that plays multiple roles in effecting adaptive responses to stress. CRF is best known as the principal hypophysiotropic hormone promoting the synthesis and secretion of pituitary corticotropin (ACTH) (1). The peptide has also been shown to be widely distributed in functionally diverse systems throughout the brain (2), and when administered centrally, it evokes indices of autonomic (3) and behavioral (4–6) activation. This has generally been construed as supporting an involvement of CRF-expressing neural systems in stress-related functions that may complement its neuroendocrine effects.

CRF initiates its biological effects through a receptor which is coupled to G_s protein and activates adenylate cyclase (7). Our laboratory has recently isolated a CRF receptor (CRF-R) cDNA by expression cloning from a Cushing adenoma (8). By homology, the CRF-R is a member of the calcitonin/vasoactive intestinal polypeptide/growth hormone-releasing factor subfamily of receptors characterized by seven membrane-spanning domains. Subsequently, we and others have cloned the homologs of the CRF-R from rat whole brain (9),

rat cerebellar (10), and mouse corticotrope (11) libraries. The human pituitary and rat brain forms differ at 12 amino acid residues, 10 of which are clustered at the N terminus and the first and second extracellular loops. Both the human and rat receptors have been shown to bind CRF with high affinity and to stimulate cAMP synthesis in vitro (8, 10).

While autoradiographic studies of CRF-binding sites (12) have complemented the available immunocytochemical literature in providing a basis for interpreting pharmacological probing of central sites of CRF action (13), ligand-binding patterns correlate only crudely with the distribution of CRF pathways (2), with some major areas of mismatch (14). One potential basis for this discrepancy derives from the recent identification of a distinct CRF-binding protein (CRF-BP), which is prominently expressed in brain, including subsets of CRF-containing pathways and/or their targets (15). The identification of this distinct high-affinity CRF-binding moiety, which is in a position to modify central and pituitary actions of CRF by autocrine or paracrine mechanisms, could limit the ability to draw inferences concerning CRF-R distributions on the basis of ligand-binding patterns alone. The present study employed hybridization histochemical methods to provide an initial overview of the cellular sites of expression of the CRF-R in rat brain and pituitary. Dual staining approaches were used to directly compare loci of CRF-R mRNA expression with those of ACTH in the pituitary and of CRF-BP in brain.

MATERIALS AND METHODS

In Situ Hybridization. Male ($n = 7$) and female ($n = 3$) adult Sprague–Dawley albino rats (200–300 g) were perfused transcardially with 4% paraformaldehyde in 0.1 M pH 9.5 sodium borate buffer, and regularly spaced series of 20- to 30-μm-thick frozen sections through brain and pituitary were taken as described (16). Radiolabeled antisense and sense (control) complementary RNA (cRNA) copies were synthesized from a full-length or N-terminal 344 bp fragment of the rat CRF cDNA subcloned in pBluescript KS vector (Stratagene). [α-^{33}P]UTP was used for probe synthesis, and in situ hybridization was performed as previously described (16, 17). cRNA probes to full-length rat CRF-R coding region were labeled to 40–60% total incorporation, and hybridization was carried out under high stringency [50% (vol/vol) formamide with final washes carried out in 0.2× SSC at 70°C (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)].

Combined *in Situ* Hybridization and Immunohistochemistry. Concurrent localization of ACTH immunoreactivity (-ir) and CRF-R mRNA in pituitaries was performed by using a modification of a procedure described by Watts and Swanson (18). This involved first applying a conventional biotin-avidin–immunoperoxidase protocol, to localize primary antibodies raised in rabbit against the rat corticotropin fragment ACTH-(23–39). Rat ACTH-(23–39) was coupled to human α-globulins with bisdiazotized benzidine, and antisera were

Abbreviations: CRF, corticotropin-releasing factor; CRF-R, CRF receptor; CRF-BP, CRF-binding protein; ACTH, corticotropin; cRNA, complementary RNA; -ir, immunoreactivity or immunoreactive.

raised in rabbits by using Freund's adjuvant as described (19). Affinity chromatography involved a column with rat ACTH-(23–39) covalently linked; elution and specificity were determined as described previously (19). Heparin sulfate at 2 mg/ml and 5% bovine serum albumin were substituted for normal goat serum in the initial incubation. Pituitaries were postfixed for 2 hr and full-length ^{33}P-labeled CRF-R mRNA probe was applied to sections for 24 hr.

A similar method was used to allow dual localization of CRF-BP-ir with CRF-R mRNA. These experiments were carried out in complete series of sections through the brains of two rats that received intracerebroventricular injection of colchicine (50 μg in 25 μl of saline) 48–72 hr prior to perfusion. Colchicine treatment has been shown previously to enhance the detectability of CRF-BP-ir in neuronal perikarya without altering the cellular sites of CRF-BP expression (15). A rabbit polyclonal antiserum raised against recombinant human CRF-BP (code 5144) was purified on a CRF affinity column and applied to tissue at a 1:3000 dilution. A ^{33}P-labeled full-length antisense cRNA probe for CRF-R mRNA was used for the subsequent *in situ* hybridization.

Northern Blot Analysis. RNA was prepared from 3-day neonate and adult rat brains by using guanadinium/CsCl and oligo(dT)–latex methods (8, 20). Three micrograms of poly(A)$^+$ RNA was run on a 1.5% formaldehyde agarose gel and a blot was hybridized with the 1.3-kb rat CRF-R mRNA probe labeled with ^{32}P by random priming (8).

RESULTS

Initial studies using probes generated from a 500-bp cDNA encoding the human CRF-R for *in situ* hybridization analysis on rat brain sections failed to reveal any specific signal. Therefore, two cRNA probes were generated from rat CRF-R cDNAs, one encoding 344 bp of the N-terminal sequence and the other a 1.3-kb *Pst* I fragment encompassing the complete coding sequence for the receptor protein. Both probes yielded identical, and widespread, patterns of CRF-R mRNA localization. Fig. 1 provides an overview of the distribution of positively hybridized cells seen when probes generated from the 1.3-kb *Pst* I fragment were used, which provided the greater sensitivity in our experiments. Labeled sense-strand cRNAs failed to show any suggestion of positive localization in complete series of sections from the same brains that displayed positive signals with antisense probes. In addition, Northern blot analysis using the full-length probe revealed a single mRNA species (2.7 kb) in poly(A)$^+$ RNA isolated from whole neonatal or adult rat brain.

Forebrain. Telencephalic structures were predominant among the forebrain sites of CRF-R mRNA expression (Fig. 1). All neocortial areas were associated with substantial CRF-R mRNA signal. This tended to be most intense over layer IV, and somewhat less so over layers II–III and VIa (Fig. 2A). In the hippocampal formation, positively hybridized cells were numerous in the hilar region of the dentate gyrus, and the pyramidal cell layer of all subfields of Ammon's horn was overlain by a moderate hybridization signal. In addition, CRF-R mRNA was detectable throughout all of the layers of the entorhinal cortex and in the pyramidal cell layer of the subicular complex. Expression of the CRF-R mRNA was seen at all levels of the olfactory pathway, including the principal projection neurons of the olfactory bulb.

Among subcortical telencephalic structures, the basal ganglia contained a low density of cells bearing a moderate hybridization signal, though receptor transcripts were more robustly expressed in functionally associated cell groups in the forebrain (substantia innominata, magnocellular preoptic nucleus, subthalamic nucleus) and midbrain (substantia nigra, ventral tegmental area). In the limbic region of the telencephalon, receptor mRNA expression in the septal region was dominated by strong and uniform signal over the

medial septal nucleus and the nucleus of the diagonal band, with lower-level signal seen diffusely over the lateral division of the bed nucleus of the stria terminalis. Within the amygdaloid complex, moderate expression over the lateral and basolateral nuclei was dominant, with the medial, cortical, and basomedial nuclei all showing lesser signal intensities. Receptor expression in the central nucleus, however, was limited to weak and sporadic labeling over its medial aspect.

CRF-R mRNA expression in diencephalic cell groups was generally low, limited in the thalamus largely to a few cell groups in the ventral tier, the parafascicular and paraventricular nuclei, and aspects of both the lateral and medial geniculate complexes. In hypothalamus, signal was generally weak and diffuse, with the dorsomedial and supramammillary nuclei and the posterior hypothalamic area containing the more focally dense accumulations of positively hybridized neurons. Among neurosecretory structures, hybridization signals were seen at low levels over the paraventricular and arcuate nuclei, and signal was not consistently detectable over the supraoptic nucleus, the anterior periventricular nucleus, or the median eminence.

Brainstem. Many of the more prominent brainstem sites of CRF-R mRNA expression (Figs. 1 and 2 *B* and *C*) could be categorized either as a major sensory relay structure or as one directly related to cerebellar function. Cell groups involved in the processing of somatic sensory information, including the dorsal column, laterodorsal and pedunculopontine tegmental nuclei, along with major trigeminal sensory structures, all comprised sites of prominent cellular CRF-R expression. Each of the vestibular nuclei contained a moderate density of positively hybridized neurons, as did multiple levels of the primary auditory path (ventral cochlear, lateral superior olivary, and lateral lemniscal nuclei); lower labeling was seen diffusely over the inferior colliculus and medial division of the medial geniculate body. In addition to the visual cortex and thalamus, CRF-R mRNA was expressed at moderate levels in the superior colliculus, including its superficial aspects, and throughout much of the visual pretectum. Somewhat surprisingly, the receptor was less widely expressed in visceral sensory systems. Although the lateral parabrachial nucleus contained a low to moderate density of positively hybridized cells (Fig. 2B), such cells were virtually lacking in the nucleus of the solitary tract (Fig. 2C), save for circumscribed expression over its central subnucleus.

In the cerebellum, cells strongly hybridized with CRF-R mRNA probe were seen in the deep nuclei, with somewhat lower signal intensities detected uniformly over the Purkinje and granule cell layers of the cerebellar cortex. A number of major pre- and postcerebellar nuclei were also found to be rich in receptor mRNA; the pontine gray, lateral reticular, and red nuclei comprised among the most prominent sites of expression that were encountered in our material. Moderate levels of CRF-R mRNA expression were seen over the perihypoglossal nuclei, while the inferior olivary complex contained only weak and intermittent hybridization signals.

Among motor nuclei, positive hybridization signals were seen principally over the compact column of the nucleus ambiguus and the facial motor nucleus. Low level signal was seen over substantial territories of the brainstem reticular core through mesencephalic, pontine, and medullary levels.

Pituitary Gland. Positive CRF-R mRNA signals of moderate intensity were localized continuously over the intermediate lobe of the pituitary and over a subset of adenohypophyseal cells (Fig. 2D). Preparations in which hybridization histochemical detection of CRF-R mRNA was combined with immunoperoxidase localization of ACTH-ir revealed above-background accumulations of silver grains over a majority of immunostained corticotropes (Fig. 3). Few grain clusters indicative of positively hybridized, non-ACTH-ir cells were detected. Because of the reduced efficiency of
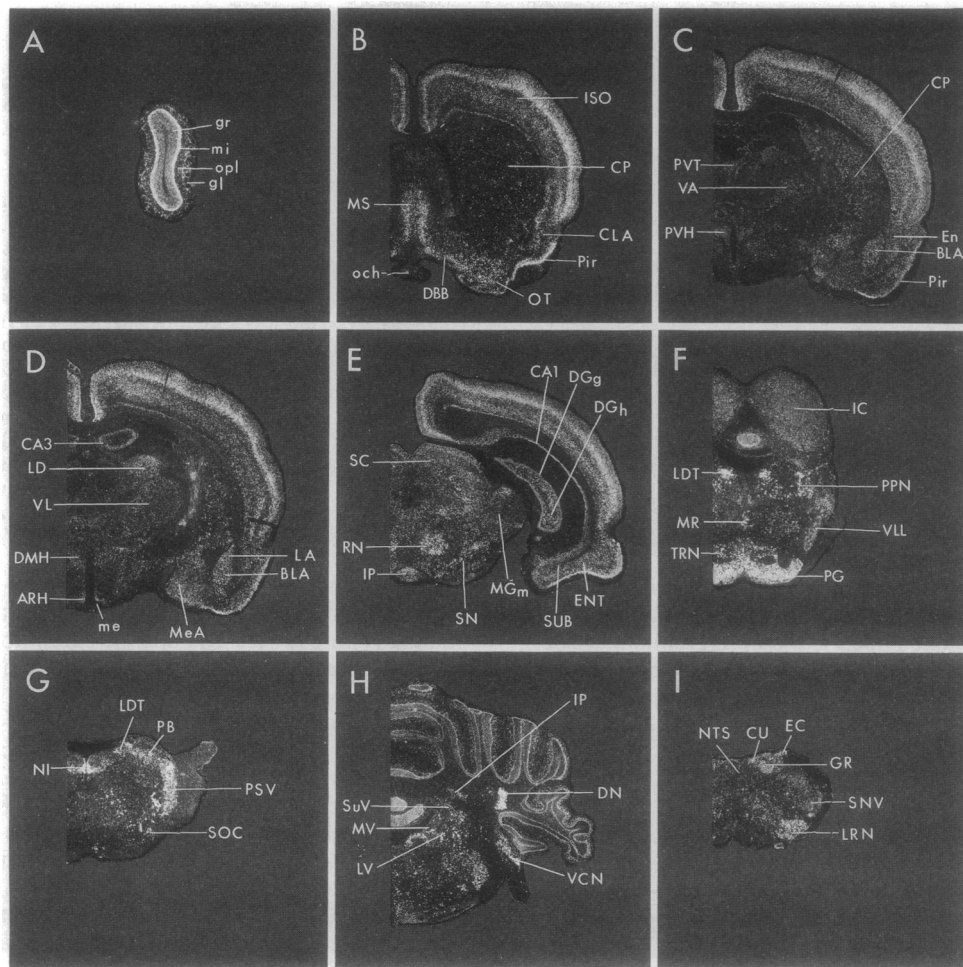
FIG. 1. Hybridization histochemical localization of CRF-R mRNA in the rat brain. A series of dark-field photomicrographs is arranged from rostral (*A*) to caudal (*I*) to show the regional distribution of neurons positively hybridized with an antisense probe generated from full-length (1.3-kb) cDNA encoding the CRF-R mRNA. (All ×1.5.) ARH, arcuate nucleus (n.); BLA, basolateral n. (amygdala); CA1, field CA1 (Ammon's horn); CA3, field CA3 (Ammon's horn); CLA, claustrum; CP, caudoputamen; CU, cuneate n.; DBB, n. of diagonal band of Broca; DGg, granule cell layer (dentate gyrus); DGh, hilar region (dentate gyrus); DMH, dorsomedial n. (hypothalamus); DN, dentate n.; EC, external cuneate n.; En, endopiriform n.; ENT, entorhinal cortex; gr, granule cell layer (olfactory bulb); GR, gracile n.; gl, glomerular layer (olfactory bulb); IC, inferior colliculus; IP, interpeduncular n.; ISO, isocortex; LA, lateral n. (amygdala); LD, laterodorsal n. (thalamus); LDT, laterodorsal tegmental n.; LRN, lateral reticular n.; LV, lateral vestibular n.; me, median eminence; MeA, medial n. (amygdala); mi, mitral cell layer (olfactory bulb); MGm, medial geniculate body (medial division); MR, median raphe n.; MS, medial septal n.; MV, medial vestibular n.; NI, n. incertus; NTS, n. of the solitary tract; och, optic chiasm; opl, outer plexiform layer (olfactory bulb); OT, n. of the optic tract; PB, parabrachial n.; PG, pontine gray; Pir, piriform cortex; PPN, pedunculopontine n.; PSV, principle sensory n., of the trigeminal nerve; PVH, paraventricular n. (hypothalamus); PVT, paraventricular n. (thalamus); RN, red nucleus; SC, superior colliculus; SN, substantia nigra; SNV, spinal trigeminal n.; SOC, superior olivary complex; SUB, subiculum; SuV, superior vestibutal n.; TRN, tegmental reticular nucleus; VA, ventral anterior n. (thalamus); VCN, ventral cochlear n.; VL, ventral lateral n. (thalamus); VLL, ventral n. of the lateral lemniscus.

hybridization in such combined staining paradigms, we cannot discount the possibility of low CRF-R mRNA expression in other anterior pituitary cell types.

Relationship to Sites of CRF-BP Expression. Because the distribution of CRF-R mRNA described above suggested partial overlap with some previously demonstrated cellular sites of CRF-BP expression (15), a similar combined immunolabeling (CRF-BP-ir) and *in situ* hybridization (CRF-R mRNA) method was applied to sections from colchicine-treated rats to identify any major area of congruence. This material revealed only limited areas of overlap, though once again this conclusion is tempered by the decrement in sensitivity of both constituent methods in such combined applications. Despite the widespread expression of both markers in neocortex, unequivocal examples of cells displaying both markers were not encountered. By contrast, robust hybridization signals were detected over a substantial fraction of CRF-BP-ir cells present in the laterodorsal and pedunculopontine tegmental nuclei (Fig. 3). Isolated examples of dou-

bly labeled cells were also seen in the piriform cortex, the olfactory bulb and tubercle, the hilar region of the dentate gyrus, and in the nuclei of the lateral lemniscus.

DISCUSSION

The present survey provides an initial overview of the cellular sites of CRF-R mRNA expression in the rat brain and pituitary. The distribution described here is in substantial agreement with the results of previous CRF radioligand-binding studies (12), although the emphases suggested by the two methods are somewhat distinct, and each technique has identified putative sites of receptor expression not revealed by using the other. A comparison of these results with the existing literature on the organization of CRF-containing terminal fields and pharmacologically defined sites of action highlight some important areas of accord, though major areas of disparity or "mismatch" remain to be reconciled (2).

The widespread expression of CRF-R mRNA in the intermediate lobe and in a sizeable subset of cells in the anterior
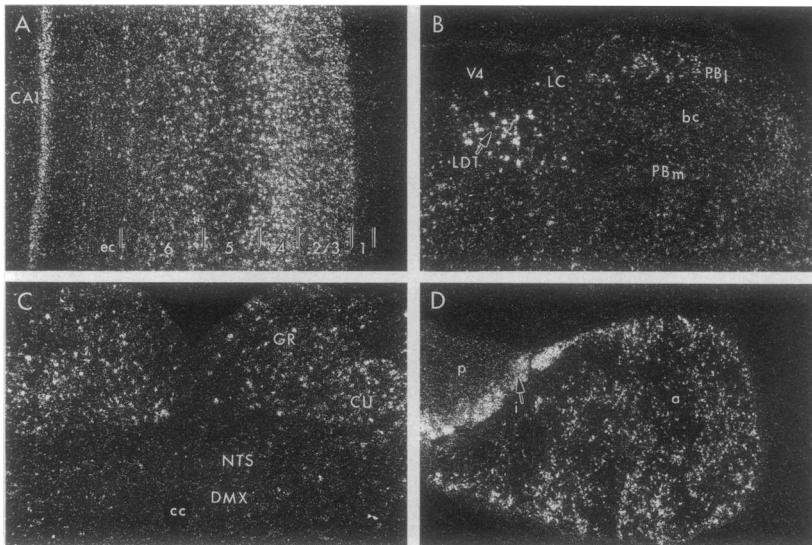
FIG. 2.   Dark-field photomicrographs to show detail of CRF-R mRNA expression in rat brain and pituitary by *in situ* hybridization. (*A* and *C*, ×40; *B* and *D*, ×25.) (*A*) Primary auditory cortex displays a strong hybridization signal over layer IV, and secondary ones over layers II/III and VI. (*B*) In the dorsolateral pons, CRF-R mRNA signal is intense over neurons of the laterodorsal tegmental nucleus (LDT), moderate over aspects of the parabrachial nucleus (PB), and at or near background levels over the locus coeruleus (LC). (*C*) In dorsomedial medulla, positively hybridized neurons are numerous in the gracile (GR) and cuneate (CU) nuclei, but not in the nucleus of the solitary tract (NTS). (*D*) The pituitary gland displays CRF-R mRNA signal that is seen uniformly over the intermediate lobe (i, arrow) and over a subset of cells in the anterior lobe (a). bc, Brachium conjunctivum; cc, central canal; DMX, dorsal motor nucleus of the vagus; ec, external capsule; p, posterior pituitary; V4, fourth ventricle.

pituitary is consistent with results of ligand-binding studies in this tissue (12). Our observation that CRF-R mRNA expression in the anterior lobe appears to be preferentially associated with corticotropes would be predicted of this receptor, given the major biological action of CRF in stimulating the

FIG. 3.   Colocalization of CRF-R mRNA and CRF-BP-ir in the pedunculopontine nucleus (*Upper*) and with ACTH-ir in the anterior pituitary (*Lower*). Shown are bright-field photomicrographs in which hybridization histochemical localization of CRF-R mRNA with ^{33}P-labeled antisense cRNA probe was superimposed on a background of avidin–biotin–immunoperoxidase localization of one of the two antigens. Reduced silver grains indicative of a positive hybridization signal appear as black dots; the immunoperoxidase staining appears as a uniform gray defining cellular boundaries. Arrows indicate examples of cells displaying both markers. (Both ×375.)

synthesis and secretion of ACTH and β-endorphin in response to stress. Only a subset of corticotropes have been found to express CRF-BP, and the extent to which such cells may coexpress the CRF-R remains to be determined.

The expression of CRF-R transcripts in structures constituting the central limb of the hypothalamo–pituitary–adrenal axis appears limited. Parvocellular neurosecretory neurons in the paraventricular nucleus of the hypothalamus (PVH) are acknowledged as the principal source of CRF-ir terminals in the median eminence, whence the peptide is conveyed via the hypophyseal–portal vasculature to the anterior lobe. We were unable to detect a positive hybridization signal over the median eminence; the fact that this region exhibits significant CRF binding (12) could be attributable to axonal transport and presynaptic localization of receptor protein. Two cell groups that project to the median eminence, the arcuate nucleus and the PVH, were found to display positive, albeit weak, hybridization signals for CRF-R mRNA. The ostensibly low level of basal receptor expression in the PVH is somewhat surprising in view of pharmacologic (21) and anatomical (22) evidence supporting the existence of CRF influences on the activity of CRF-expressing neurons in this cell group.

Outside of the neuroendocrine system, the central distribution of CRF-R mRNA described here conforms well with the results of autoradiographic studies of the binding patterns of iodinated CRF analogs. The somewhat greater relative strength and/or more expansive distribution of ligand binding described in several major areas, including the basal ganglia and thalamus, could be attributable to binding by receptors localized to presynaptic terminals and/or by an alternative CRF-R form or subtype not recognized by our probes. The other known high-affinity CRF-binding moiety, the CRF-BP, is not expressed at all extensively in either of the above-mentioned areas (15). On the other hand, hybridization data reveal some novel putative sites of receptor expression in the midbrain (e.g., substantia nigra, red nucleus) and pons (e.g., pedunculopontine and laterodorsal tegmental nuclei, nucleus incertus), and they suggest disproportionately high levels in areas related to sensory and cerebellar function. The extent to which the relative strength of receptor protein expression may mirror that of its cognate mRNA in each of these areas remains to be determined.

The high degree of correspondence between the present data and previously described patterns of radioligand binding suggest the CRF binding can be attributable in large measure to binding by receptors synthesized locally. While in some instances, notably in the pituitary, cerebellum, and olfactory system, this is in register with data from complementary
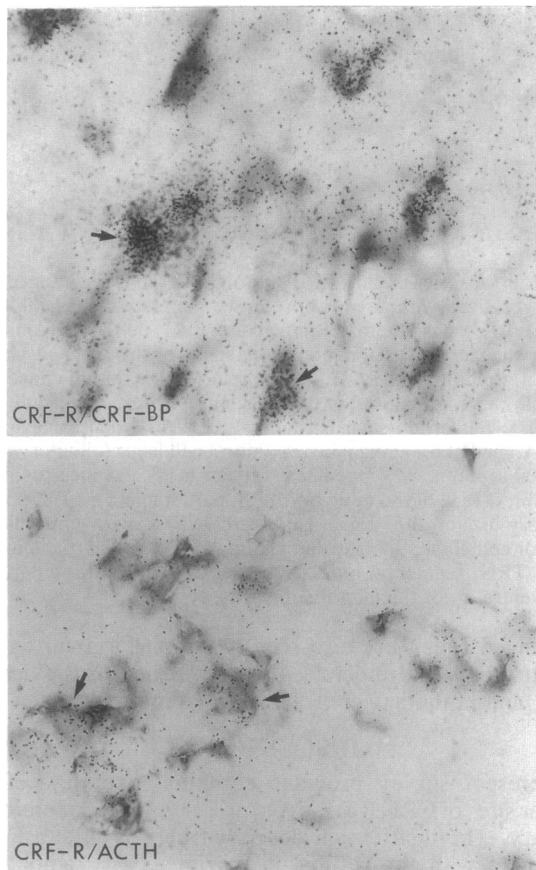
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immunohistochemical (23) and/or functional studies (24), in others it serves to accentuate areas of discord or mismatch. In the rat neocortex, for example, CRF-ir has been detected principally in bipolar interneurons concentrated in layers II and III, and in sparse and diffusely distributed axons. This contrasts starkly with the widespread distribution of cortical neurons expressing CRF-R mRNA, which is focally most dense over layer IV. Similarly, the available immunohistochemical evidence provides little support for a substantial CRF-ir input to the somatic and trigeminal sensory structures identified here as major sites of CRF-R mRNA expression.

CRF is recognized as one of a number of neuropeptides which are prominently expressed in a core group of structures intimately involved in central autonomic and neuroendocrine regulation. These areas include the nucleus of the solitary tract, catecholaminergic cell groups of the ventrolateral medulla and pons, parabrachial nucleus, locus coeruleus, central nucleus of the amygdala, bed nucleus of the stria terminalis, and multiple cell groups in the hypothalamus and preoptic region (2). In general, CRF-R mRNA expression in these areas is relatively low. Moreover, two of these structures, the locus coeruleus and the central amygdaloid nucleus, have been among those more prominently implicated as central sites of CRF action relevant to stress-related autonomic and/or behavioral responses (25, 26), and yet they display little capacity for CRF-R mRNA expression or CRF binding.

These areas of mismatch between the distribution of cellular sites of CRF-R mRNA expression and CRF-ir terminal fields or sites of action could be attributable, in part, to axonal transport of receptors or to the relative insensitivity of immunohistochemical methods in detecting CRF antigens in axons and terminals. Some support for the latter possibility derives from the results of recent studies in the rat olfactory (17) and auditory (27) systems, and in primate neocortex (28), where focused immunohistochemical analyses have been effective in revealing a distribution of CRF-ir projections more in register with the patterns of receptor binding and mRNA expression. Furthermore, we cannot exclude the possibility that an additional ligand(s) for the CRF-R may exist. The family of structurally related peptides to which CRF belongs includes members presently known to exist only in nonmammalian species. Two of these, urotensin and sauvigine, are bound with moderate to high affinity by the CRF-R and exert biological actions in mammalian brain.

The recent identification of a CRF-BP, which is expressed in brain and pituitary, presented a potential basis by which to pinpoint sites of CRF binding that might not be explicable on the basis of CRF-R expression patterns. Although a few structures, such as the dorsal tegmental and lateral septal nuclei, were identified that reportedly exhibit substantial CRF-binding activity (12) and CRF-BP expression (15), but displayed little detectable CRF-R mRNA, the present results provide little clear evidence of such. The limited resolution achievable in ligand-binding preparations leaves open to question whether the relatively small number of discrete sites at which CRF-BP expression appears to be unique, or far more prominent than that of CRF-R, actually make up significant regions of CRF binding. The distributions of the binding protein and receptor share a number of general features, including a broad distribution in cortical regions, the limbic region of the telencephalon, and a number of sensory relay nuclei. Even in major areas of overlap, dual labeling for CRF-BP-ir and CRF-R mRNA identified few individual neurons displaying both markers. Notable exceptions were the pedunculopontine and laterodorsal tegmental nuclei, where numerous doubly labeled cells were identified. These represent potential sites at which the binding protein might competitively limit, or otherwise modify, ligand–receptor interactions.

In summary, we have described a pattern of CRF-R mRNA expression in rat brain and pituitary that is similar to, though more expansive than, that of regions reported to bind CRF, and that appears to show only limited overlap with loci of CRF-BP expression. The distribution of CRF-R transcripts in pituitary appears fully compatible with the principal neuroendocrine action of CRF. In brain, however, the general pattern of receptor expression is more suggestive of primary involvements in cortical, cerebellar, and multiple modalities of sensory information processing than in the stress-related autonomic, neuroendocrine, and behavioral responses for which CRF is better known. It remains for efforts aimed at localization of receptor protein, identification of alternative ligands for the receptor, isolation of novel receptor forms or subtypes, and alterations of receptor expression due to stress and changes in hormonal milieu to clarify the role of the CRF-R in the central nervous system.

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