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Localization of Novel Corticotropin-Releasing Factor Receptor (CRF_2) mRNA Expression to Specific Subcortical Nuclei in Rat Brain: Comparison with CRF_1 Receptor mRNA Expression

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Corticotropin-releasing factor (CRF) is the primary factor involved in controlling the release of ACTH from the anterior pituitary and also acts as a neurotransmitter in a variety of brain systems. The actions of CRF are mediated by G-protein coupled membrane bound receptors and a high affinity CRF receptor, CRF_1, has been previously cloned and functionally characterized. We have recently isolated a cDNA encoding a second member of the CRF receptor family, designated CRF_2, which displays approximately 70% homology at the nucleotide level to the CRF_1 receptor and exhibits a distinctive pharmacological profile. The present study utilized *in situ* hybridization histochemistry to localize the distribution of CRF_2 receptor mRNA in rat brain and pituitary gland and compared this with the distribution of CRF_1 receptor expression. While CRF_1 receptor expression was very high in neocortical, cerebellar, and sensory relay structures, CRF_2 receptor expression was generally confined to subcortical structures. The highest levels of CRF_2 receptor mRNA in brain were evident within the lateral septal nucleus, the ventromedial hypothalamic nucleus and the choroid plexus. Moderate levels of CRF_2 receptor expression were evident in the olfactory bulb, amygdaloid nuclei, the paraventricular and suraoptic nuclei of the hypothalamus, the inferior colliculus and 5-HT–associated raphe nuclei of the midbrain. CRF_2-expressing cells were also evident in the bed nucleus of the stria terminalis, the hippocampal formation and anterior and lateral hypothalmic areas. In addition, CRF_2 receptor mRNA was also found in cerebral arterioles throughout the brain. Within the pituitary gland, CRF_2 receptor mRNA was detectable only at very low levels in scattered cells while CRF_1 receptor mRNA was readily detectable in anterior and intermediate lobes. This heterogeneous distribution of CRF_1 and CRF_2 receptor mRNA suggests distinctive functional roles for each receptor in CRF-related systems. The CRF_1 receptor may be regarded as the primary neuroendocrine pituitary CRF receptor and important in cortical, cerebellar and sensory roles of CRF. The anatomical distribution of CRF_2 receptor mRNA indicates a role for this novel receptor in hypothalamic neuroendocrine, autonomic and general behavioral actions of central CRF.

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[Key words: corticotropin-releasing factor (CRF), stress, CRF receptors, mRNA expression, brain,* in situ *hybridization]

The 41 amino acid peptide corticotropin-releasing factor (CRF) is the primary hypothalamic factor involved in controlling the synthesis and release of adrenocorticotropin hormone (ACTH) from the anterior pituitary and consequently acts as a key regulator of hypothalamo-pituitary-adrenal (HPA) function (Vale et al., 1981). In addition, an abundance of evidence indicates a central neurotransmitter role for this peptide and CRF-producing cells are found in a variety of systems throughout the brain (Swanson et al., 1983). Central administration of CRF results in general behavioral and autonomic activation implicating CRF as a central mediator of stress-related responses (Fisher, 1993; Koob et al., 1993b). Based on these observations, and accumulated clinical studies (Holsboer et al., 1984; Kathol et al., 1989; Nemeroff et al., 1991), CRF is recognized as a putative pathophysiological factor in stress-related disorders such as depression and anxiety.

The actions of CRF are mediated by G-protein coupled membrane-bound receptors which have been extensively characterized in brain and pituitary gland (De Souza and Kuhar, 1986; De Souza, 1987; Grigoriadis and De Souza, 1989). Autoradiographic analysis of these sites indicates a broad brain distribution in general concordance with the distribution of CRF pathways (De Souza et al., 1985). A high affinity CRF receptor, CRF_1, has been recently cloned and functionally characterized by a number of independent laboratories (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993). This site displays high affinity for CRF and related peptides, and is positively linked to adenylate cyclase. Analysis of CRF_1 receptor mRNA expression in rat brain indicates a pattern of expression which is broadly complementary to the distribution of CRF binding sites (Potter et al., 1994). However, it is significant that CRF_1 receptor mRNA is absent in several brain regions in which CRF effects are evident.

Using a degenerate sequence polymerase chain reaction (PCR) paradigm, we have recently isolated a cDNA clone encoding a second member of the CRF receptor family (Lovenberg et al., 1995). This receptor, designated CRF_2, displays approximately 70% identity at the nucleotide level to the CRF_1 site over the coding region. As with the CRF_1 receptor, when expressed *in vitro*, the CRF_2 receptor stimulates cAMP production in response to CRF and related nonmammalian peptides, sauvagine and urotensin I. However, the rank order of potency of these compounds at the CRF_2 receptor (sauvagine $>$ urotensin $\geq$ rat/human CRF $>$ ovine CRF) differs from that at the CRF_1 receptor

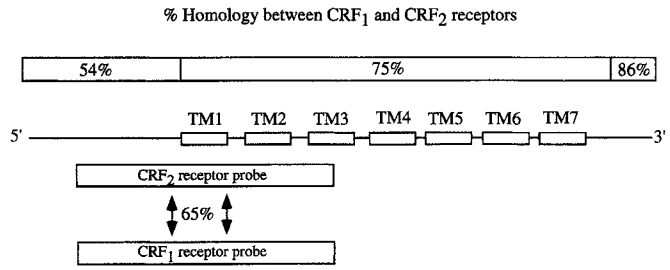

Figure 1. Schematic illustration of nucleotide sequence homology between CRF_1 and CRF_2 receptors across the coding region of the receptors. *Lower bars* indicate the region of the receptors against which CRF_1 and CRF_2 cRNA probes were designed.

at which r/h CRF is the most potent ligand. This pharmacological heterogeneity suggests important functional differences between CRF_1 and CRF_2 receptors in mediating the biological activity of CRF and/or related peptides *in vivo*. In the present study we have employed *in situ* hybridization histochemistry to precisely localize the distribution of cells expressing CRF_2 mRNA in rat brain and compared this with the expression pattern for the CRF_1 receptor. This analysis suggests circuit-specific roles for CRF_2 receptors in brain.

Materials And Methods

Animals. Male Sprague–Dawley rats (200–220 gm) were used for mapping studies. Prior to sacrifice, animals were housed in a 12 hr light/dark cycle with food and water provided ad libitum.

Riboprobe design. CRF_2 cRNA riboprobe was produced from a 460 bp cDNA fragment of the CRF_2 receptor subcloned into pBluescriptSK+ (Stratagene, La Jolla) and linearized with XbaI. CRF_1 probe was synthesized from a 460 bp 5′ fragment of CRF_1 cDNA in pBluescriptSK+, linearized with XbaI. Both CRF_2 and CRF_1 riboprobes were directed against the 5′ region of their respective receptors, covering the sequence up to the third presumed transmembrane region (Fig. 1). The approximate nucleotide homology between the two probes is 65% in this region. Importantly, in preliminary experiments, cRNA probes directed against the 3′ region of the CRF_2 receptor apparently labeled both CRF_1 and CRF_2 receptor mRNAs whereas the two mRNA species could be clearly separated by 5′ specific probes under similar hybridization conditions. Thus, it seems necessary to utilize 5′ probes for subtype-specific labeling. For both probes, specificity was confirmed by absence of signal in sections labeled with sense probe and sections pretreated with RNase prior to hybridization with antisense (cRNA) probe. CRF cRNA antisense probes were synthesized from a 770 bp fragment of CRF cDNA subcloned into a pGEM3Z vector (courtesy Dr. Robert Thompson, University of Michigan). Riboprobes were produced using either T3 or T7 transcription systems in a standard labelling reaction mixture consisting of: 1 μg of linearized plasmid, 5 × transcription buffer, 125 μCi of ^{35}S-UTP or ^{33}P-UTP, 150 μM NTPs, 12.5 mM dithiothreitol, 20 U of RNase inhibitor, and 6 U of the appropriate polymerase. The reaction was incubated at 37°C for 90 min, labeled probe being separated from free nucleotides over Sephadex G-50 spin columns.

In situ *hybridization histochemistry.* Dissected tissue was frozen in isopentane cooled to −42°C and subsequently stored at −80°C prior to sectioning on a cryostat. Slide-mounted tissue sections were then stored at −80°C. Sections were removed from storage and placed directly into 4% buffered paraformaldehyde at room temperature. After 60 min, slides are rinsed in isotonic phosphate-buffered saline (10 min) and treated with proteinase K (1 μg/ml in 100 mM Tris/HCl, pH 8.0) for 10 min at 37°C. Subsequently, sections underwent successive washes in water (1 min), 0.1 M triethanolamine (pH 8.0, plus 0.25% acetic anhydride) for 10 min and 2 × SSC (0.3 mM NaCl, 0.03 mM sodium citrate, pH 7.2) for 5 min. Sections were then dehydrated through graded alcohols and air dried. Postfixed sections were hybridized with 1.0 × 10^6 dpm ^{35}S-UTP-labeled riboprobes in hybridization buffer containing 75% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate buffer (pH 7.4), 1 × Denhardt's solution, 0.1 mg/ml yeast tRNA, and 10 mM dithiothreitol in a total volume of 30 μl. The diluted probe was applied to sections on a glass coverslip and hybridized overnight

Table 1. Semiquantitative evaluation of CRF_1 and CRF_2 receptor mRNA distribution in rat brain and pituitary gland

Anatomical region	mRNA abundance CRF_1	mRNA abundance CRF_2
Telencephalon		
Olfactory bulb:		
External granular layer	++	−
Internal granular layer	++++	++
Mitral cell layer	++++	−
Ependyma	++	+++
Accessory olfactory nucleus	++	++
Frontal cortex (superficial)	+++	−
Frontal cortex (deep)	+++	−
Cingulate cortex (superficial)	+++	−
Cingulate cortex (deep)	+++	−
Lateral septum (ventral)	+	++++
Lateral septum (intermediate)	+	++++
Medial septum	++	−/+
Bed nucleus of the stria terminalis (medial)	++	++
Amygdala		
Basolateral nucleus	++++	−/+
Medial nucleus	++++	++
Posterior cortical	+	+++
Hippocampus		
CA1	++	++
CA3/4	++++	++
Dentate gyrus	++	++
Entorhinal cortex	++	++
Diencephalon		
Hypothalamus		
Paraventricular nucleus	−/+	++
Supraoptic nucleus	+	+++
Lateral hypothalamus	+	+
Dorsomedial hypothalamus	+++	−
Ventromedial hypothalamic nucleus	+	++++
Medial geniculate nucleus	++	−/+
Mesencephalon		
Superior colliculus (superficial layer)	+++	+
Interpeduncular nucleus	++++	+++
Dorsal raphe nucleus	+	++
Caudal linear nucleus	+	+++
Red nucleus	++++	−
Pons/medulla		
Inferior colliculus	++	++
Lateral dorsal tegmental nucleus	++++	−
Locus coeruleus	−	−
Cerebellar cortex	++++	−/+
Pontine gray	++++	−/+
Motor trigeminal nucleus	++++	−
Sensory trigeminal nucleus	+++	−/+
Choroid plexus	−	++++
Pituitary gland		
Anterior lobe	+++	−/+
Intermediate lobe	+++	−/+
Posterior lobe	−	−

CRF_1 and CRF_2 mRNA abundance for each anatomical region was determined from optical density measurements. Density values for each parameter are presented according to their respective percentile distributions: ++++ (>75%), very dense; +++ (<75%, >50%), dense; ++ (<50%, >25%), moderate; + (<25%, >10%), low; −/+ (<10%), scattered cells.

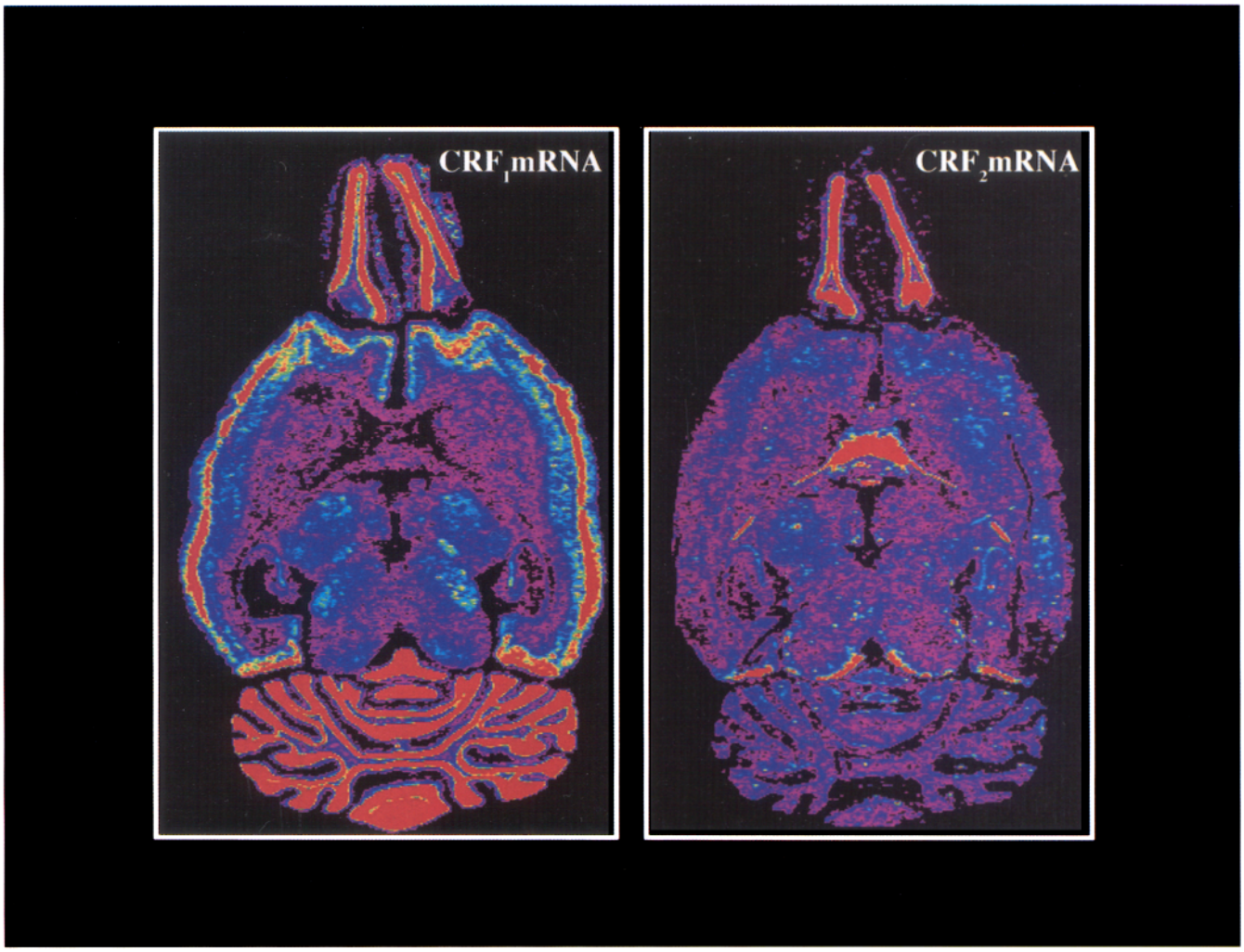

Figure 2. Color-coded digitized images of CRF_1 and CRF_2 receptor mRNA expression in adjacent horizontal brain sections. Regions exhibiting high levels of mRNA expression are coded in *red* and *orange* while the lowest levels of expression are coded in *blue*.

at 55°C in a humid environment. Posthybridization, sections were washed in 2 × SSC for 5 min and then treated with RNase A (200 μg/ml in 10 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl) for 60 min at 37°C. Subsequently, sections were washed in 2 × SSC for 5 min, 1 × SSC for 5 min, 0.1 × SSC for 60 min at 70°C, 0.5 × SSC at room temperature for 5 min and then dehydrated in graded alcohols and air dried. For signal detection, sections were placed on Kodak Bio Max x-ray film and exposed for the required length of time or dipped in photographic emulsion (Amersham LM-1) for high resolution analysis. Autoradiograms were analysed using automated image analysis (DAGE camera/Mac II/IMAGE program). Briefly, anatomical regions of interest were interactively selected and mean optical density measurements determined from at least three coronal sections. Background signal was determined from an area of section in which labeling was undetectable. Dipped sections were examined using a Zeiss Axioscope.

Results

CRF_2 probe selection

As illustrated in Figure 1, the CRF_2 cRNA probe utilized in the present studies was synthesized from a 460 bp 5′ fragment of CRF_2 cDNA. Preliminary *in situ* hybridization studies using cRNA probes encompassing the 3′ portion of the receptor (bearing high homology to the CRF_1 receptor) afforded an anatomical distribution which was inconsistent with that obtained using 5′-specific riboprobes. The pattern of labeling was, however, consistent with *both* CRF_1 and CRF_2 receptor mRNA distribution. The high sequence homolgy between CRF_1 and CRF_2 receptors thus necessitates the use of 5′-containing riboprobes for specific hybridization of CRF_2 *or* CRF_1 receptor subtype mRNA.

Anatomical distribution

The comparative anatomical distribution of CRF_2 and CRF_1 mRNA was determined in adjacent coronal brain sections. Table 1 summarizes the semiquantitative analysis of the data.

As illustrated in horizontal section (Fig. 2), the distribution of CRF_2 receptor mRNA clearly differs from that of CRF_1, exhibiting a distinct sub-cortical pattern. While CRF_1 mRNA expression was high in a range of telencephalic structures, CRF_2 receptor expression exhibited a more anatomically specific pattern including the lateral septal region (LS), the bed nucleus of the stria terminalis (BNST), the amygdaloid area, and the olfactory bulb (Fig. 3, Table 1). The contrast in expression patterns between CRF receptor subtypes was particularly evident within the septal region: CRF_2 mRNA expression was very high in the lateral septal nuclei but very low in the medial septum, the septal nucleus where CRF_1 mRNA abundance was most evident (Fig. 3*B*). The distribution of CRF_2 receptor mRNA within the LS was, however, heterogeneous; very high levels of expression were evident within both the intermediate and ventral subnuclei

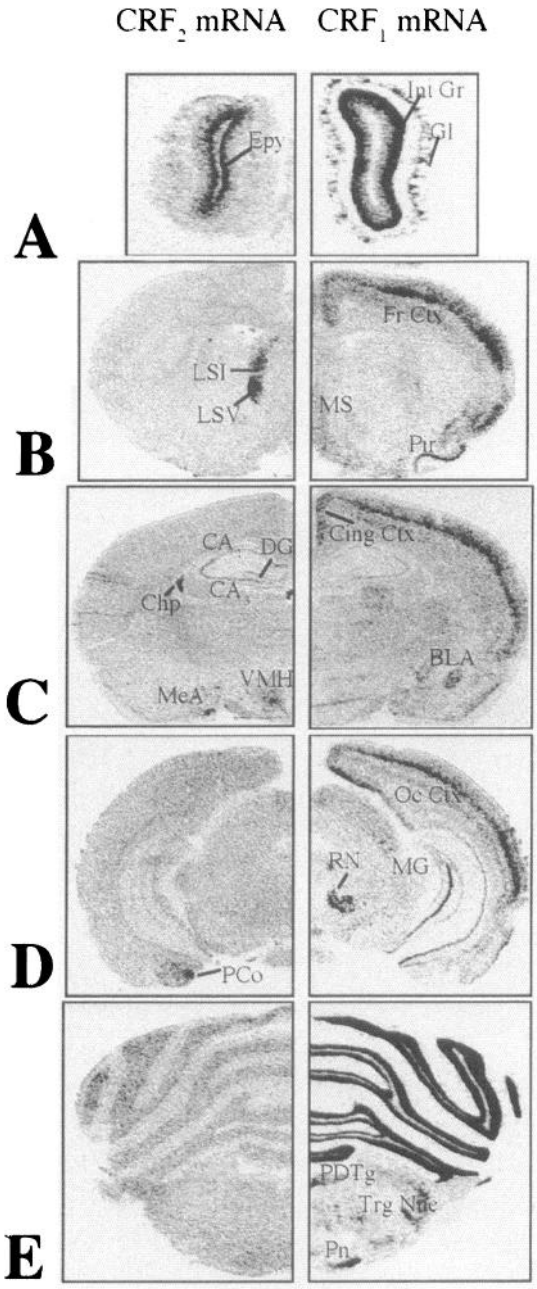

Figure 3. Rostro-caudal (*A–E*) distribution of CRF_2 receptor mRNA (left hemisphere) and CRF_1 receptor mRNA (right hemisphere) in digitized coronal brain sections. *Epy*, Ependymal layer of olfactory bulb; *Int Gr*, internal ganule cell layer of olfactory bulb; *Gl*, granule cell layer of olfactory bulb; *LSI*, lateral septal nucleus (intermediate part); *LSV*, lateral septal nucleus (ventral part); *MS*, medial septal nucleus; *Fr Ctx*, frontal cortex; *Pir*, piriform cortex; CA_1, field CA_1 (Ammon's horn); CA_3, field CA_3 (Ammon's horn); *DG*, dentate gyrus; *Chp*, choroid plexus; *MeA*, medial amygdaloid nucleus; *VMH*, ventromedial hypothalamic nucleus; *Cing Ctx*, cingulate cortex; *BLA*, basolateral amygdaloid nucleus; *PCo*, posterior cortical amygdaloid nucleus; *RN*, red nucleus; *Oc Ctx*, occipital cortex; *MG*, medial geniculate nucleus; *PDTg*, posterior dorsal tegmental nucleus; *Trg Nuc*, trigeminal nuclei; *Pn*, pontine gray.

with only an ocassionally labeled cell evident in the dorsal subdivision (Fig. 4). Within both the intermediate and ventral regions of the LS the level of CRF_2 receptor expression exhibited an apparent rostro-caudal gradient, with a smaller percentage of labelled cells detected in the caudal aspects of both areas.

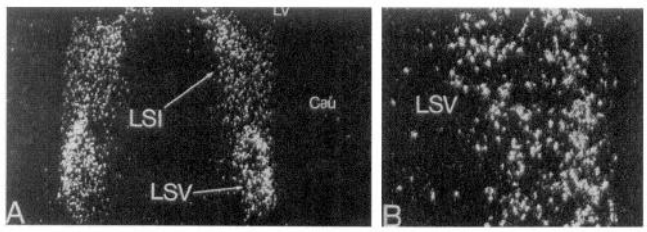

Figure 4. Dark-field photomicrographs of cells hybridized with ^{35}S-cRNA CRF_2 probe in (*A*) the intermediate (*LSI*) and ventral (*LSV*) lateral septal nuclei. At high resolution (*B*) note the high level of CRF_2 receptor expression in cells in the ventral lateral septum. *Cau*, caudate; *LV*, lateral ventricle.

At this level, some scattered CRF_2-expressing cells were also evident in both the vertical and horizontal limbs of the diagonal band. Again, contrasting the higher levels of CRF_1 mRNA found in these regions (Fig. 3). Differential patterns of CRF receptor subtype expression were also evident within the olfactory bulb (Fig. 3*A*). Here, CRF_1 receptor expression was particularly high in the internal granule cell and mitral cell layers with lower levels detectable in the external granule layer and ependyma. However, most cells expressing CRF_2 receptors were found lining the ventricle within the ependymal area and the internal granule cell layer (Fig. 3*A*). Both CRF_1 and CRF_2 receptors were also expressed in the accessory olfactory nucleus.

Both CRF_2 and CRF_1 receptor mRNA expression were evident in the BNST, particularly in the medial aspect, and the amygdaloid area. Within the BNST, CRF_2 receptor expression appeared to be lower in the lateral regions where the highest abundance of CRF-expressing cells were found (Fig. 5*A,B*). CRF_1 receptor expression was found in both medial and lateral divisions of the BNST (Fig. 5*C*). Within the amygdala the highest levels of CRF_2 receptor expression were evident in the cortical and medial amygdaloid nuclei with less expression in the basolateral nucleus (Fig. 3*C*). In complementary fashion, CRF_1 receptor expression was very high in the basolateral area but low in the cortical amygdala (Fig. 6). Both CRF_1 and CRF_2 receptor mRNA expression were unremarkable in the central nucleus. Within the hippocampal formation, CRF_2 receptors were generally expressed in low to moderate levels in pyramidal cells of CA subfields and granule cells of the dentate gyrus. However, scattered cells with high levels of CRF_2 expression were evident in non-granule cell layers in ventral dentate gyrus (Fig. 7). CRF_1 receptor expression was most abundant in the pyramidal cell layer of $CA_{3/4}$ with moderate levels evident in CA_1. However, CRF_1 receptor expression was apparently absent in the dorsal dentate gyrus (Fig. 7). Interestingly, higher levels of CRF_1 expression were evident in the ventral hippocampus compared to the dorsal aspect (Fig. 3*C,D*). Cells expressing both CRF_1 and CRF_2 were found throughout entorhinal cortex.

The distribution of CRF_2 receptor transcripts in diencephalic structures was confined mainly to the hypothalamus where labelled cells were evident in preoptic, anterior, and tuberal regions. The levels of CRF_2 mRNA expression found in the ventromedial hypothalamic nuclei (VMH) were among the highest detected in brain (Fig. 8). Both dorsal and ventral aspects of VMH displayed high levels of CRF_2 mRNA relative to other brain regions although CRF_2 mRNA expression was most evident in the dorsomedial division. At this level, however, CRF_1 receptor expression was predominantly localized to the dorso-

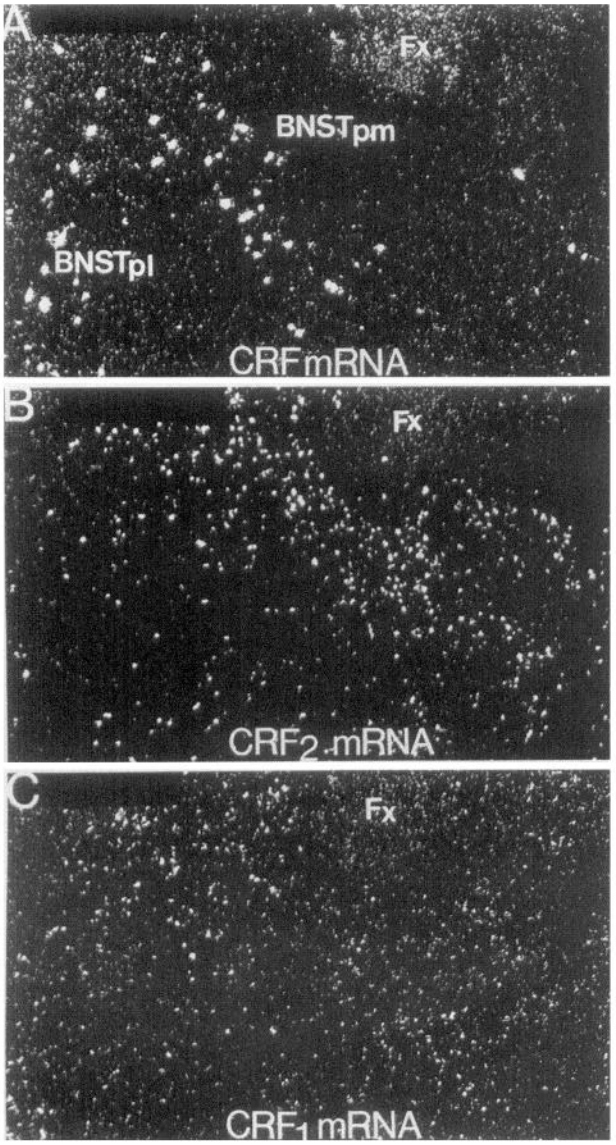

Figure 5. Dark-field photomicrographs of cells hybridized with: *A*, ^{35}S cRNA CRF probe; *B*, ^{35}S cRNA CRF_2 probe; and *C*, ^{35}S cRNA CRF_1 probe in adjacent coronal sections through the bed nucleus of the stria terminalis (*BNST*). In *A* note the higher concentration of CRF-expressing cells in the posterolateral area (*BNSTpl*) while in *B* CRF_2 receptor expression is predominantly localized to the medial aspects of the nucleus (*BNSTpm*). In *C* CRF_1 receptor mRNA expression is evident in both medial and lateral aspects of the nucleus. *Fx*, fornix.

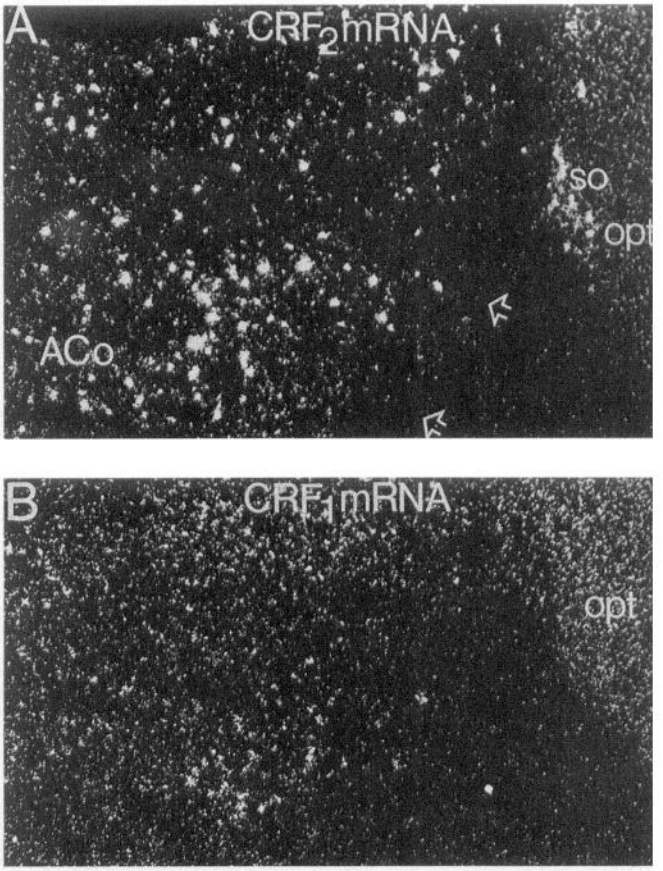

Figure 6. Dark-field photomicrographs of cells hybridized with: *A*, ^{35}S cRNA CRF_2 probe, and *B*, ^{35}S cRNA CRF_1 probe in the anterior cortical amygdaloid nucleus (*ACo*). In *A* not the high level of CRF_2 receptor mRNA expression in cells throughout the nucleus while in *B* CRF_1 receptor expression is comparable to background signal. CRF_2 receptor mRNA expression is also evident within the supraoptic nucleus in *A*, an area where CRF_1 receptor expression is undetectable (*B*). *opt*, optic tract; *arrows* in *A* indicate section edge.

medial hypothalamic nucleus (DMH) with only a limited number of cells labeled in the VMH. In anterior hypothalamus, CRF_2 mRNA was localized to highly expressing cells in the supraoptic nucleus (SO) and medial areas of the paraventricular nucleus (PVN) (Figs. 9, 10). Within the PVN, CRF_2 mRNA appeared to be expressed in medial parvocellular cells, partially coinciding with CRF mRNA expression (Fig. 10*A*). This raises the interesting possibility that CRF_2 receptors may act as autoreceptors in selective cells in this nucleus. In both the SO and PVN, the number of cells expressing CRF_1 mRNA was extremely low (Figs. 9, 10). Cells expressing CRF_2 mRNA were also evident throughout the anterior and lateral hypothalamic areas, the suprachiasmatic nucleus and in the medial preoptic area (MPA).

In the midbrain, CRF_2-expressing cells were evident throughout nuclei biochemically characterized as serotonin-containing nuclei. Strongly hybridizing cells were localized along the midline in the dorsal raphe nucleus (DR) the caudal linear nucleus and in the lateral aspects of the central gray (Fig. 11). More ventrally, CRF_2 receptor expression was also detectable in the median raphe (MR) and the interpeduncular nucleus (IPN), especially within the rostral subnucleus (Fig. 11). With the exception of IPN, CRF_1 receptor mRNA levels were generally low in all of these areas. At this level, CRF_2-expressing cells were also evident in clusters in the lateral and dorsal regions of the inferior colliculus. A low level of CRF_1 mRNA expression was also present in the inferior colliculus. However, higher levels of CRF_1 mRNA were found in the visually receptive fields of the superior colliculus where CRF_2 receptor expression was very low. This differential expression pattern was repeated in other sensory relay structures of the brainstem. Thus, while CRF_1 receptor mRNA abundance levels were very high in tegmental nuclei and sensory trigeminal areas, CRF_2 receptor expression was limited to a few scattered cells in these structures (Table 1). Similarly, within the cerebellum, CRF_1 receptor mRNA levels were very high in Purkinje and granule cell layers while CRF_2 receptor

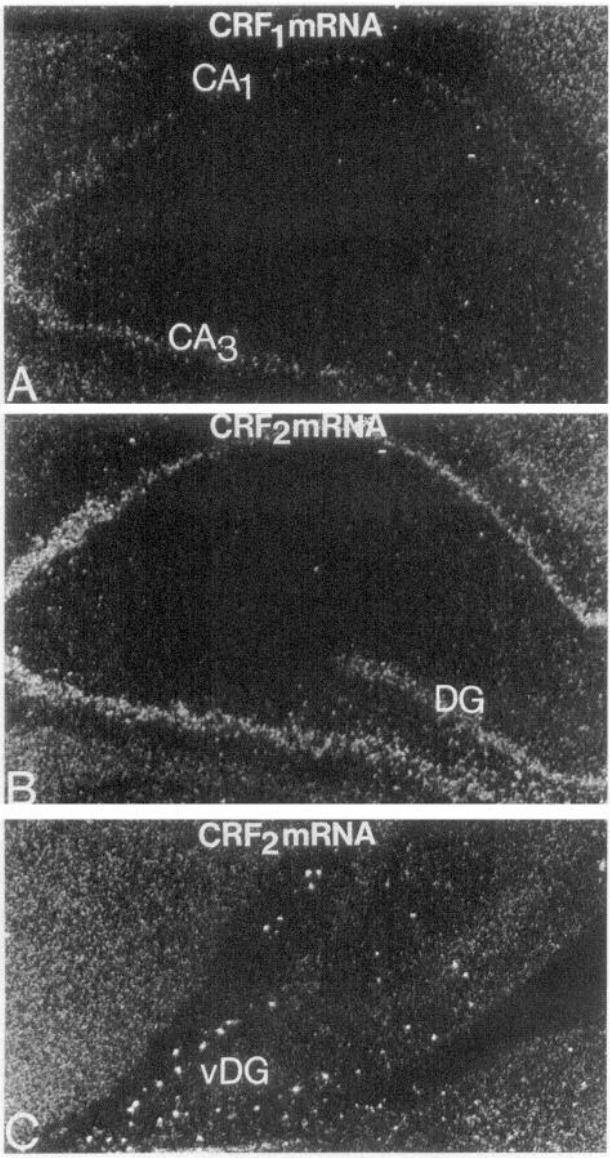

Figure 7. Dark-field photomicrographs of cells hybridized with: *A*, ^{35}S cRNA CRF_1 probe, and *B* and *C*, ^{35}S cRNA CRF_2 probe in the hippocampal formation. Within dorsal hippocampus, both CRF_1 and CRF_2 receptor expression was relatively low. However, within the ventral hippocampus (*C*), cells expressing high levels of CRF_2 receptor mRNA were evident in the dentate gyrus and subiculum. *, Emulsion artefact.

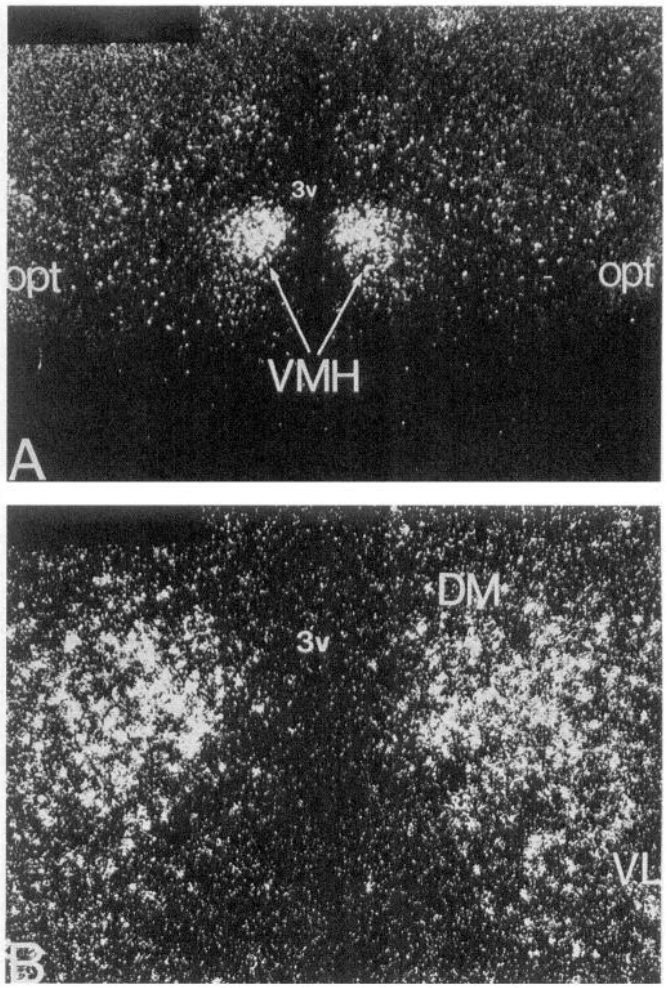

Figure 8. Dark-field photomicrographs of cells hybridized with ^{35}S cRNA CRF_2 probe in the ventromedial hypothalamic nucleus (*VMH; A, B*). At high resolution (*B*), note the high level of CRF_2 receptor expression in both the dorsomedial (*DM*) and ventrolateral (*VL*) aspects of the nucleus. *3v*, Third ventricle; *opt*, optic tract.

mRNA was present at very limited levels in granule cells (Fig. 3*E*).

Within non-neuronal structures, a very high level of CRF_2 receptor expression was evident in the choroid plexus of the third, fourth and lateral ventricles (Fig. 12). In addition, cerebral arterioles consistently exhibited CRF_2 mRNA at all brain levels examined (Fig. 13). CRF_1 mRNA was detectable at near background levels in these structures. In the pituitary gland, CRF_1 expression was detectable in both anterior and intermediate lobes with particularly high expression in clusters within the anterior lobe (Fig. 14). Within the anterior lobe, CRF_2 receptor expression was detectable only in scattered cells (Fig. 14).

Discussion

The present *in situ* hybridization studies indicate that at least two CRF receptor subtypes, CRF_1 and CRF_2, are expressed in mammalian rat brain. The heterogeneous anatomical distribution patterns of CRF_1 and CRF_2 mRNA expression suggests distinct functional roles for each receptor in CRF-related CNS circuits. While CRF_1 receptor expression was most abundant in neocortical, cerebellar, and sensory relay structures, CRF_2 receptor expression was generally localized to specific subcortical structures, including the lateral septum and various hypothalamic nuclei. The distribution of cells expressing CRF receptor subtypes is, however, generally concordant with previous immunocytochemical studies of CRF pathways in brain (Sawchenko and Swanson, 1990).

Within the forebrain, the highest levels of CRF_2 receptor expression were found in the lateral septal nuclei. The lateral septum, by virtue of widespread reciprocal connections throughout the brain, is implicated in a variety of physiological processes. These range from higher cognitive functions such as learning and memory to autonomic regulation, including food and water intake (De France, 1976). In addition, the septum plays a central role in classical limbic circuitry and is thus important in a variety of emotional conditions including fear and aggression. The lateral septal nuclei receive CRF-containing afferents from rostral hypothalamic regions, particularly the anterior hypothalamic

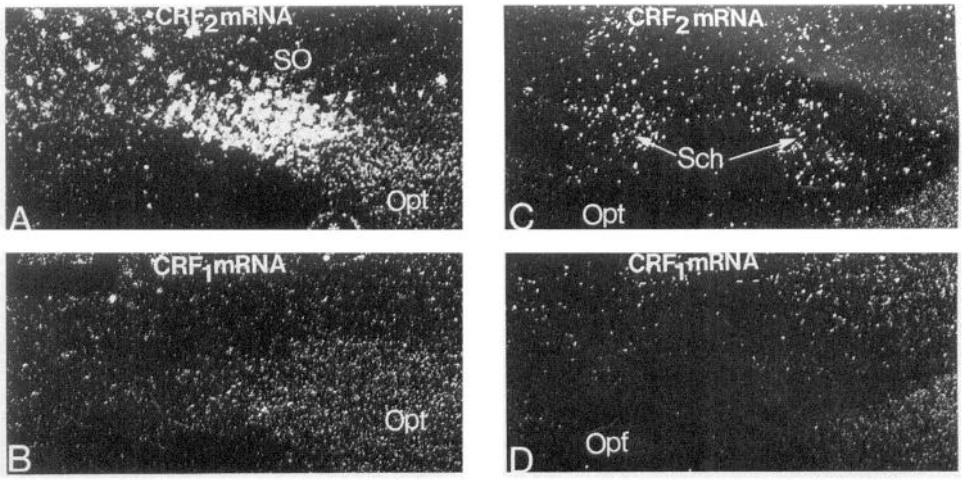

Figure 9. Dark-field photomicrographs of cells hybridized with ^{35}S cRNA CRF_2 probe (*A, C*) and ^{35}S cRNA CRF_1 probe (*B, D*) in adjacent coronal sections through the supraoptic nucleus (*SO*) and suprachiasmatic nucleus (*Sch*). Note the absence of CRF_1 receptor expression in either nucleus (*B, D*). *Opt*, Optic tract; *, labeled arteriole.

area (Sakanaka et al., 1988). Interestingly, the majority of these CRF-like immunoreactive fibres are found in the most lateral aspects of the LSV and in the LSI (Sakanaka et al., 1988), septal subnuclei exhibiting the highest levels of CRF_2 receptor mRNA (Fig. 4). 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. The principal GABAergic neurons of the lateral septum provide inhibitory input to hypothalamic regions as well as amygdaloid nuclei (Jakab et al., 1991) and receive excitatory glutamatergic input from the hippocampal formation (Joels and Urban, 1984). The lateral septum thus acts as both an integrator of limbic circuitry and an interface between telencephalic and diencephalic areas. The high level of CRF_2 receptor expression in this area suggests a role for CRF_2 receptors in modulating limbic circuitry at the level of septal activity. Exploration of the specific involvement of these sites in the general activating effects of exogenously administered CRF (Koob et al., 1993a) awaits the development of specific receptor antagonists.

In agreement with previous *in situ* hybridization studies (Potter et al., 1994), we have found a general lack of CRF_1 receptor expression in hypothalamic nuclei. Previous reports of CRF_1 receptor mRNA expression in the VMH and PVN may relate to the use of 3′ containing riboprobes and/or low stringency hybridization conditions which resulted in detection of both CRF_1 and CRF_2 mRNA (Wong et al., 1994). From the present studies it is clear that CRF_2 receptor mRNA was evident throughout the rostro-caudal extent of the hypothalamus while CRF_1 receptor expression was limited. The difference in CRF receptor subtype expression levels was particularly evident within the paraventricular nucleus where CRF_2 receptor expression was readily detectable while CRF_1 receptor mRNA was present only in scattered cells. The distribution of cells expressing CRF_2 receptor mRNA within the PVN coincides with the cellular distribution of CRF mRNA (Fig. 10) suggesting a possible autoreceptor role for CRF_2 receptors in this nucleus. The CRF neurons of the PVN play a classical hypophysiotropic role in controlling ACTH release from the anterior pituitary (Wiegand and Price, 1980) and as such are central to the control of the mammalian hypothalamo-pituitary-adrenal system. In addition to this stress axis-related role, subpopulations of PVN CRF neurons, particularly within the dorsal parvocellular region and ventral aspect of the medial parvocellular region (mpv), project to autonomic cell groups in the brainstem and spinal cord (Sawchenko, 1987). The pos-

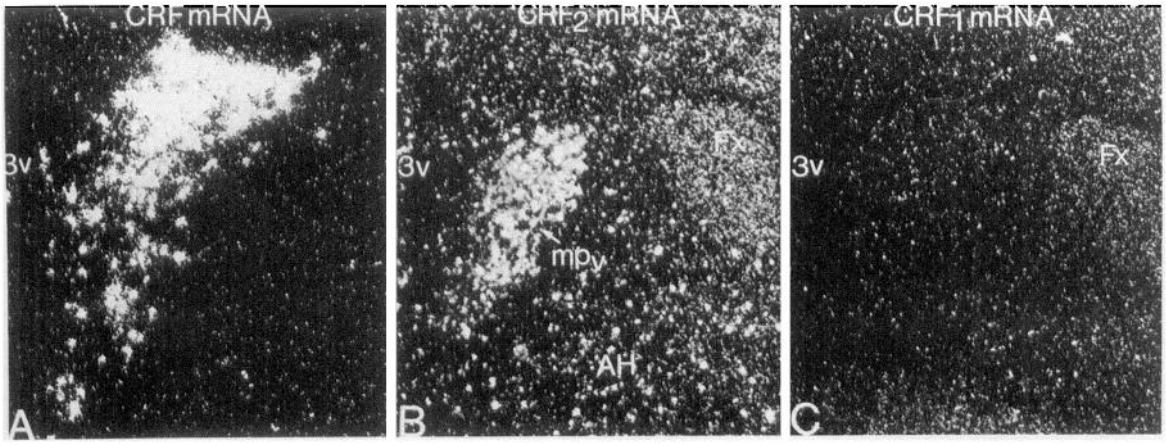

Figure 10. Dark-field photomicrographs of cells hybridized with: *A*, ^{35}S cRNA CRF probe; *B*, ^{35}S cRNA CRF_2 probe; and *C*, ^{35}S cRNA CRF_1 probe in adjacent sections through the paraventricular nucleus of the hypothalamus. In *A*, CRF expressing cells are evident throughout the medial and dorsal aspects of the paraventricular nucleus. In *B* note that CRF_2 receptor expression is most prominant in the medial parvocellular area (mp_v) with only scattered labeled cells evident in the dorsal subdivision. CRF_1 receptor expression is unremarkable in both subdivisions (*C*). *3v*, Third ventricle; *Fx*, fornix.

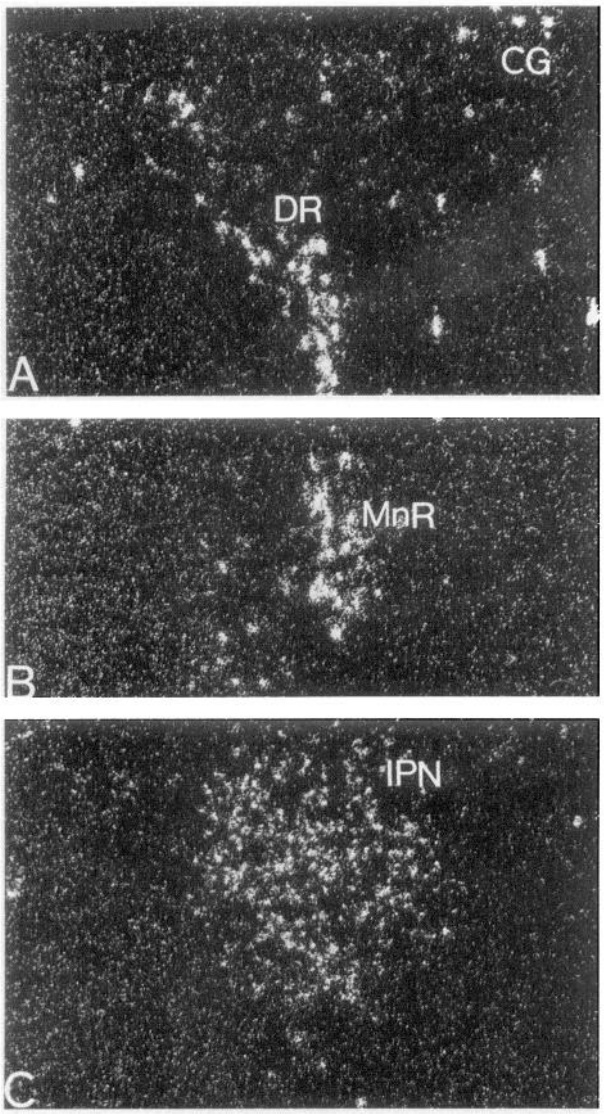

Figure 11. Dark-field photomicrographs of cells hybridized with ^{35}S cRNA CRF_2 probe in: *A*, dorsal raphe (*DR*) and central gray (*CG*); *B*, median raphe (*MnR*); and *C*, interpeduncular nucleus (*IPN*).

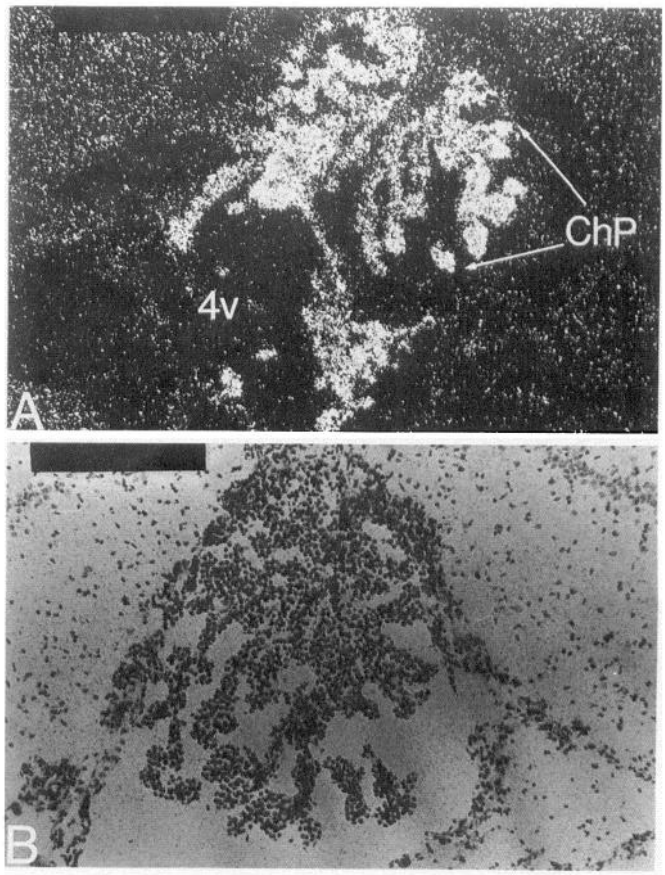

Figure 12. *A*, CRF_2 receptor mRNA expression in the choroid plexus (*ChP*). *B*, An adjacent Nissl stained section. *4v*, Fourth ventricle.

sibility that CRF_2 receptors may act to selectively regulate hypophysiotropic and/or autonomic-related CRF neurons requires further double-labeling studies. However, the high level of CRF_2 receptor expression within the mpv is certainly suggestive of a presynaptic role for CRF_2 receptors in modulating autonomic-related CRF projection neurons. With regard to HPA axis activity, we may speculate that PVN CRF_2 receptors could also act to modulate CRF neurosecretory neurons in response to stress. In essence, a short-loop feedback role. Such receptors may be relevant to the proposed role of CRF in positive ultrashort loop feedback effects on stress-induced ACTH release (Ono et al., 1985). In this regard, the comparatively lower affinity of CRF for the CRF_2 receptor (Lovenberg et al., 1995) may be of physiological design to allow recruitment of CRF_2 receptors only under conditions of prolonged CRF release. However, it remains possible that CRF_1 receptor expression may be "induced" in PVN neurons under stressful conditions and subsequently participate in mediating local CRF effects (Rivest, 1994). In addition, bearing in mind the high affinity of the nonmammalian CRF-like peptides urotensin and sauvagine for the CRF_2 receptor (Lovenberg et al., 1995), it remains possible that additional endogenous mammalian ligands for the CRF_2 receptor exist. However, direct administration of CRF into the PVN, under nonstressful conditions, does in itself produce a spectrum of endocrine, autonomic, and behavioral responses (Menzaghi et al., 1993), including anxiogenic, anorexic, and locomotor effects. Based on the present data it would appear that these effects of locally injected CRF are CRF_2 receptor-mediated.

A selective expression of CRF_2 receptor mRNA was also evident in the magnocellular neurosecretory neurons of the supraoptic nucleus. Whether these sites are associated with oxytocin or vasopressin neurons remains to be established. However, in view of the putative association of CRF_2 receptor expression with CRF neurons in the PVN, it is relevent that a subset of SO neurons also synthesize CRF (Kawata et al., 1983). The presence of CRF_2 receptors in both SO and PVN neurons indicates that these sites may act to influence hypothalamic input to both the anterior and posterior pituitary. Within the pituitary, however, CRF_1 receptor expression predominates over CRF_2 expression in both the intermediate and anterior lobes. Thus, in terms of HPA axis activity, CRF_2 receptors may mediate CRF effects at the level of the hypothalamus while CRF_1 receptors are responsible for CRF-induced changes in ACTH release in pituitary corticotropes.

Within the caudal hypothalamus, CRF_1 and CRF_2 receptors exhibited mutually exclusive patterns of mRNA distribution: CRF_1 receptor mRNA being abundant in the dorsomedial nucle-

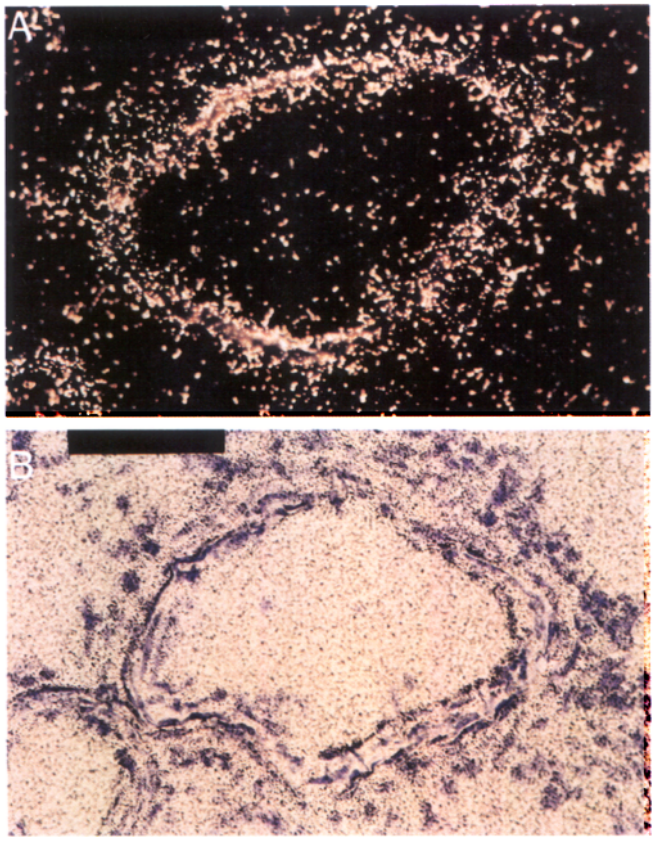

Figure 13. *A*, High power darkfield image of CRF_2 receptor mRNA expression in a cerebral arteriole. *B*, Bright-field Nissl-stained section of arteriole shown in *A*. Note the characteristic muscular arteriole wall in *B*.

us but low within the VMH, while CRF_2 receptor mRNA expression was very high within the VMH but undetectable within the DMH. CRF-containing fibers originating in the corticomedial amygdala and subiculum terminate within the VMH (Sakanaka et al., 1986). Afferents from the VMH in turn innervate the septum, BNST, and amygdala as well as brainstem regions (Simerly, 1995). Micoinjection of CRF into the VMH is associated with changes in both autonomic outflow and gastrointestinal function (Brown and Fisher, 1990; Tache et al., 1990). The high level of CRF_2 receptor expression in the VMH implicates this CRF receptor subtype as a terminal or somato-dendritic regulator of these CRF-related physiological functions. Moreover, dysregulation of CRF_2 receptors in this locus, or the PVN, may participate in the proposed role of central CRF systems in the development of obesity/anorectic syndromes (Krahn and Gosnell, 1989).

In addition to CRF involvement in the development of eating disorders, a large body of evidence exists to implicate this neuropeptide in the pathophysiology of affective diseases such as anxiety and depression. For example, CRF injected into the rodent locus coeruleus produces an anxiogenic response while benzodiazepine anxiolytics have been shown to reduce CRF concentrations in the same nucleus (Owens et al., 1991). In clinical studies of major depression, patients have been found to exhibit signs of CRF hypersecretion, including increased CRF concentrations in CSF, increased HPA activity, a blunted ACTH response to CRF and pituitary and adrenal hypertrophy (Owens and Nemeroff, 1993). In view of the stimulatory role of CRF in HPA activity, it remains possible that the hypercortisolemia observed in depression results from increased central CRF drive. The possibility that hyperactivity of brain CRF circuits may contribute to the symptomatology of depressive illness is supported by preclinical studies in both rodents and nonhuman primates.

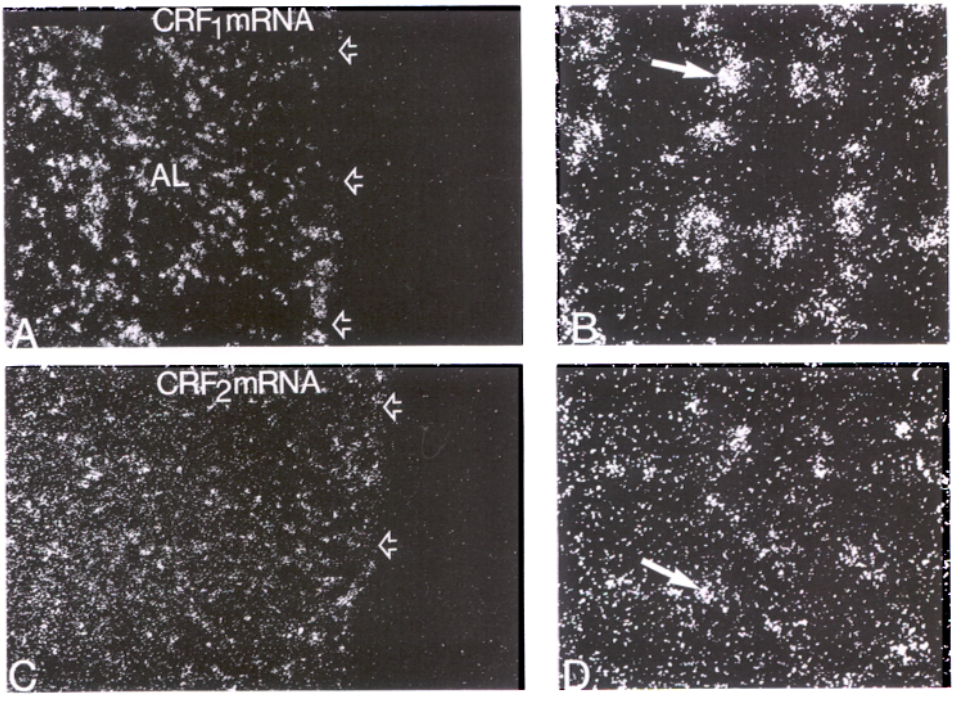

Figure 14. Dark-field photomicrographs of CRF_1 receptor mRNA expression (*A*, *B*) and CRF_2 receptor mRNA expression (*C*, *D*) in the anterior lobe (*AL*) of the pituitary gland. In *A*, note the clustering of cells expressing CRF_1 mRNA presumably reflecting the distribution of pituitary corticotropes while CRF_2 mRNA expression is present only in scattered cells (*C*). At high resolution, prominent accumulation of silver grains are evident over anterior lobe cells hybridized with ^{35}S cRNA CRF_1 probe, *arrow* in *B*, while only weak accumulations of silver grains were evident over cells hybridized with ^{35}S cRNA CRF_2 probe, *arrow* in *D*).

In both species, central administration of CRF produces a spectrum of behavioral responses reminiscent of human depressive illness (Kalin, 1990; Koob and Britton, 1990). While the specific underlying mechanisms by which CRF evokes such behavioral responses remain largely undefined , it is likely that modulation of brain limbic circuitry and the participation of specific populations of CRF neurons are involved (Rainnie et al., 1992). In this regard, the present study provides an anatomical basis for the involvement of *both* CRF_1 and CRF_2 receptors in mediating limbic CRF effects. While the CRF_2 receptor may be regarded as the predominant hypothalamic CRF receptor, both CRF_1 and CRF_2 receptors were localized to classical limbic structures such as the amygdaloid complex, the hippocampus and the septal nuclei.

In addition to neuroendocrine abnormalities, a convincing body of data indicates dysfunction in central serotonergic activity in depressive illness. From postmortem studies, it is clear that serotonin metabolism and specific receptor subtypes are altered in some brain regions in depressed patients (Meltzer, 1989; Yates et al., 1990). Drugs which inhibit 5-HT metabolism, inhibit serotonin uptake from the synapse or act to directly stimulate serotonin receptors are all effective antidepressants (Peroutka and Snyder, 1980; Traber and Glaser, 1987). Thus, as both the HPA axis and serotonergic system are implicated in affective disease, it is likely that interactions between these two systems may be relevant to the pathophysiology and pharmacotherapy of depression (Chalmers et al., 1993). In this context, the present data indicates a selective expression of CRF_2 receptor mRNA in midbrain serotonergic cell body nuclei. Both dorsal and median raphe nuclei exhibited CRF_2 mRNA as well as cells in 5-HT–associated regions of the interpeduncular nucleus and central grey (Fig. 11). As the raphe nuclei receive CRF-ergic input from forebrain regions (Sawchenko and Swanson, 1990) any CRF-induced modulation of serotonergic activity is likely to be CRF_2 receptor mediated. The physiological importance of such an interaction remains to be determined, however, it does provide an anatomical and biochemical basis for central "stress"-related modulation of serotonergic function and a basis for theories of stress-induced affective disorders.

In addition to neuronal localization the present study also indicates a high level of CRF_2 receptor expression in both the choroid plexus and cerebral arterioles. The presence of signal in both structures may be indicative of an endothelial cell localization. The CRF_2 cRNA probe used in the present studies did not differentiate between the two apparent splice forms of the CRF_2 receptor, $CRF_{2\alpha}$ and $CRF_{2\beta}$, (Lovenberg et al., 1995). However, preliminary data indicates that the $CRF_{2\beta}$ form of the receptor may predominate in blood vessels. Interestingly, the $CRF_{2\beta}$ receptor is also expressed in peripheral tissues such as heart, lung and skeletal muscle where it may putatively act to mediate vascular effects of CRF (T. W. Lovenberg, D. T. Chalmers, L. Changlu, and E. B. De Souza, unpublished observations). The local functional role of CRF_2 receptors in cerebral vessels requires further investigation but presumably relates to CRF-induced modulation of cerebral blood flow. Such sites may be relevant to both stress-induced cerebrovascular dysfunction, such as migraine, and the reported ability of CRF receptor antagonists to block neuronal damage in ischaemia models (Lyons et al., 1991; Strijbos et al., 1994).

In summary, we have identified a differential cellular distribution of CRF_2 and CRF_1 receptor mRNA in rat brain and pituitary gland. This distribution suggests that the CRF_1 receptor is the primary neuroendocrine pituitary CRF receptor and plays a dominant role in the cortical, cerebellar, and sensory roles of CRF in brain. The regional anatomical distribution of CRF_2 receptor mRNA indicates a role for this receptor in relation to hypothalamic neuroendocrine, autonomic, and behavioral actions of brain CRF. The presence of CRF_2 receptor mRNA in the hypothalamic PVN and medial and cortical amygdaloid regions may indicate an autoreceptor role for this site in selective circuits. Further investigation of CRF_2 receptor function in brain will not only further our understanding of central CRF systems but provide a basis for the development of CRF receptor subtype-specific therapeutics.

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