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Research Report

Distribution and expression of CRF receptor 1 and 2 mRNAs in the CRF over-expressing mouse brain

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ARTICLE INFO

Article history:
Accepted 5 December 2005
Available online 19 January 2006

Keywords:
Receptor plasticity
Corticotropin-releasing factor receptor 1 and 2
In situ hybridization
CRF over-expressing mouse

ABSTRACT

Corticotropin-releasing factor (CRF) acts through CRF 1 and CRF 2 receptors (CRF1, CRF2). To test the hypothesis that CRF controls the expression of these receptors in a brain site- and receptor-type specific manner, we studied CRF1 mRNA and CRF2 mRNA expressions in mice with central CRF over-expression (CRF-OE) and using in situ hybridization. CRF1 and CRF2 mRNAs appear to be differentially distributed across the brain. The brain structures expressing the receptors are the same in wild-type (WT) and in CRF-OE mice. We therefore conclude that chronically elevated CRF does not induce or inhibit expression of these receptors in structures that normally do not or do, respectively, show these receptors. However, from counting cell body profiles positive for CRF1 and CRF2 mRNAs, clear differences appear in receptor expression between CRF-OE and WT mice, in a brain-structure-specific fashion. Whereas some structures do not differ, CRF-OE mice exhibit remarkably lower numbers of CRF1 mRNA-positive profiles in the subthalamic nucleus (–38.6%), globus pallidus (–31.5%), dorsal part of the lateral septum (–23.5%), substantia nigra (–22,8%), primary somatosensory cortex (–18.9%) and principal sensory nucleus V (–18.4%). Furthermore, a higher number of CRF2 mRNA-positive profiles are observed in the dorsal raphe nucleus (+32.2%). These data strongly indicate that central CRF over-expression in the mouse brain is associated with down-regulation of CRF1 mRNA and up-regulation of CRF2 mRNA in a brain-structure-specific way. On the basis of these results and the fact that CRF-OE mice reveal a number of physiological and autonomic symptoms that may be related to chronic stress, we suggest that CRF1 in the basal nuclei may be involved in disturbed information processing and that CRF2 in the dorsal raphe nucleus may play a role in mediating stress-induced release of serotonin by CRF.

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doi:10.1016/j.brainres.2005.12.034

1. Introduction

Corticotropin-releasing factor (CRF) is a main regulator of neuroendocrine, autonomic and behavioral responses and is particularly involved in the control of adaptation to stressful conditions (Vale et al., 1981; Fisher, 1989; Holsboer et al., 1995; Nemeroff, 1996). It has a widespread distribution in the mammalian brain, in both hypothalamic and extrahypothalamic areas (Swanson et al., 1983; Valentino et al., 1992), where it acts by binding to two receptors, CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2) (Chalmers et al., 1995). CRF1 is ubiquitously expressed, e.g. in the olfactory bulb, cerebral cortex, globus pallidus (GP), red nucleus (RN), pontine gray (Pn), substantia nigra (SN), sensory and motor trigeminal nuclei and cerebellum (for complete distribution, see Van Pett et al., 2000) and is assumed to be primarily involved in sensory information processing and control of motor activity (Potter et al., 1994; Van Pett et al., 2000). CRF2 has a more restricted distribution, e.g. in the lateral septal nuclei (LS), ventromedial hypothalamic nuclei (VMH), amygdala, dorsal raphe nucleus (DR) and the bed nucleus of the stria terminalis (BNST) (Van Pett et al., 2000). This clearly differential distribution of CRF1 and CRF2 implicates that these receptors exert different functions that are controlled by different mechanisms. Although these control mechanisms are largely unknown, especially work on rat has indicated that they may involve stressor-specific up- and down-regulations of CRF receptor expression. In rat, acute stress results in increased CRF1 mRNA expression in the hypothalamic paraventricular nucleus (PVN) and the supraoptic nucleus (SON) (Luo et al., 1994; Makino et al., 1995, 1997; Rivest et al., 1995; Bonaz and Rivest, 1998; Imaki et al., 2001), whereas after chronic stress CRF1 mRNA is decreased in the frontal cortex and increased in the hippocampus and in the PVN, but less strongly in the latter than after acute stress (Makino et al., 1995; Iredale et al., 1996; Bonaz and Rivest, 1998; Brunson et al., 2002).

There are indications that CRF controls CRF1 and CRF2 plasticity, either directly or by controlling the action of other factors such as corticosteroids and that the nature of this regulation may differ among CRF1 and CRF2 receptors and among brain structures (Luo et al., 1994; Brunson et al., 2002). For example, in rat, exogenous CRF can stimulate transcription of CRF1 in the PVN (Imaki et al., 1996; Mansi et al., 1996), and CRF administration to juvenile rats increases CRF1 mRNA in the frontal cortex and hippocampus but not CRF2 expression in the basomedial amygdala (BLA) and VMH (Brunson et al., 2002). As to corticosteroids, chronic corticosterone administration or adrenalectomy results in decreased CRF1 mRNA in the rat PVN (Makino et al., 1995, 1997), whereas extrahypothalamic regions such as the amygdala and the BNST are unaffected by these treatments (Makino et al., 1995). On the other hand, in the VMH CRF2, mRNA expression increases by acute and chronic corticosterone treatment but is reduced after adrenalectomy, whereas it remains unaffected in the PVN (Makino et al., 1997, 1998).

Although these data suggest that CRF1 and CRF2 receptors are regulated in a receptor-specific and brain-specific way, the involvement of CRF and other stress regulatory factors in controlling CRF receptor expressions needs further elucidation because up until now data have been derived from studies involving a variety of stressors (e.g. chronic, acute, environmental, pharmacological) hampering generalized conclusions and have been mainly concerned with restricted parts of the rat brain. In the present study, we focus on the primary role of CRF in the regulation of CRF1 and CRF2 mRNA expressions throughout the mouse brain. For this purpose, we have used a mouse model of central CRF over-expression (CRF-OE mouse) (Groenink et al., 2002; Dirks et al., 2002a,b). Central over-expression of CRF in this animal is associated with a chronically increased level of bioactive CRF in the hypothalamus and increased CRF immunoreactivity in the PVN and in extrahypothalamic areas such as the BNST and the central nucleus of the amygdala (CeA) (Dirks et al., 2002a). In these animals, CRF is expressed in a large number of additional brain structures, such as the thalamus and the basal nuclei, which do not contain any CRF in wild-type (WT) mice (Swanson et al., 1983). Furthermore, the CRF-OE mouse exhibits down-regulation of the CRF-related peptide, urocortin 1 (Ucn1) in the Edinger–Westphal nucleus (E–WN), which is a factor assumed to play an important role in stress adaptation responses (Skelton et al., 2000b; Kozicz et al., 2004). This animal model shows hypercortisolism, adrenal gland hypertrophy and dexamethasone nonsuppression (Groenink et al., 2002), aberrant autonomic activities such as increased thermogenesis and accelerated cardiovascular activity (Dirks et al., 2002a) and impaired sensory information processing associated with reduced behavioral reactivity to environmental stimuli (Dirks et al., 2002b) and reduced locomotor activity (Groenink et al., 2003).

This study aims to test the hypothesis that CRF plays a role in controlling CRF1 and CRF2 mRNA expressions in the mouse, in a receptor- and brain-area-specific fashion. To this end, we have used a quantitative in situ hybridization approach to compare CRFR mRNA expression patterns in WT with those in the CRF-OE mice brain. By relating our results to what is known about the aberrant physiological and behavioral characteristics of the CRF-OE mouse, in the Discussion section, some suggestions will be made as to the physiological and pathophysiological aspects of CRF1 and CRF2 receptor regulations, in some distinct brain areas.

2. Results

2.1. *General remarks and observations*

In both WT and CRF-OE mice, hybridization signals were seen throughout the brain, in various brain areas as identified on the basis of the coordinates in the mouse brain atlas of Paxinos and Franklin (2001). For CRF1 mRNA, the major expressing areas were the cerebral cortex, hippocampus, dorsal part of the lateral septal nucleus, medial septal nucleus, olfactory bulb, globus pallidus, red nucleus, principal sensory nucleus V, pontine gray,

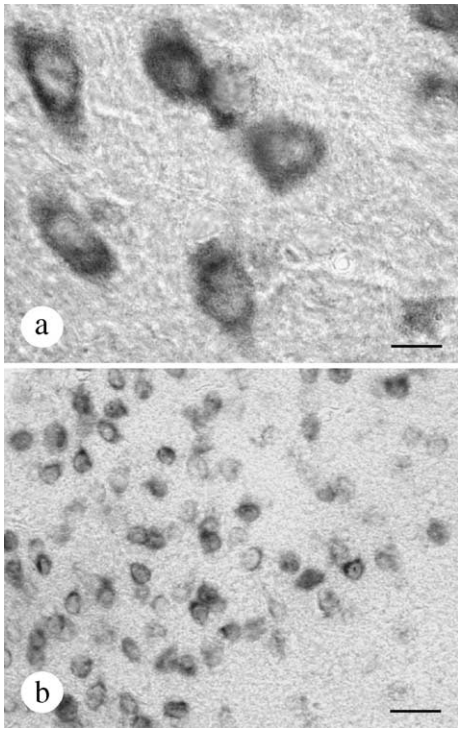

Fig. 1 – In situ hybridization of CRF2 mRNA in perikarya in the lateral septum of a WT mouse (a). Detail, showing neurons with different staining intensities (b). Scale bar a: 250 μm, b: 100 μm.

cerebellum, subthalamic nucleus, substantia nigra pars compacta and pedunculopontine nucleus. For CRF2 mRNA, major expression was in the medial amygdala, the intermediate part of the lateral septal nucleus, the posterodorsal region of the bed nucleus of the stria terminalis, the ventromedial nucleus of the hypothalamus and in the dorsal and median raphe. No other elements in the brain, like glial cells, or blood vessels, were found positive. No appreciable background staining was noticeable, and also with the sense probes (control), no hybridization signal was seen. The hybridization signal was always located in the neuronal perikarya (Fig. 1a), with variable staining intensity (Fig. 1b).

We assessed both receptor mRNA expressions by counting the numbers of stained (hybridization-positive) cell body profiles of sectioned neurons. Since it was not the aim of our study to quantify the amount of mRNA in an absolute sense but only to test for a relative difference in expressions between the two, equally treated, mouse genotypes, we have chosen for the relatively simple method of profile counting. The results confirm that this method provides the requested answers with adequate reproducibility. CRF1 and CRF2 receptor mRNAs show different distribution patterns, which, however, do not differ between WT and CRF-OE mice. On the other hand, for some brain structures, the quantification demonstrates clear differences between the two genotypes whereas other structures did not differ between WT and CRF-OE mice. Below, we will describe the expressions of both receptor types in detail for a number of brain areas selected on the basis of technical criteria (well-delineation of the structure, ease of identification of individual perikarya).

2.2. *CRF1 mRNA distribution in WT and CRF-OE mouse brain*

Looking at the average number of neuronal cell bodies with a positive hybridization signal in Table 1, it is remarkable that all brain areas showed a lower number of CRF1 mRNA-positive cell bodies in the CRF-OE mouse brain, but studying these data into more detail by means of statistical analysis, a more differential picture appears. No significant difference was found in the numbers of cell bodies between WT and CRF-OE mice in the three subdivisions of the hippocampus (CA1, CA3, DG), the medial septal nucleus, the pedunculopontine nucleus, the Pn (Figs. 2a, b), the RN and the interposed cerebellar nucleus. However, in

Table 1 – Number of neurons in brain structures with their Bregma coordinates (Paxinos and Franklin, 2001), expressing corticotropin-releasing factor receptor 1 (CRF1) mRNA in WT and CRF-OE mouse brains (n = 7)

Brain area	Bregma (mm)	WT	CRF-OE	t test
Medial septal nucleus	0.62	101.9 ± 9.2	87.4 ± 9.6	ns
Primary somatosensory cortex	0.86	82.0 ± 3.0	66.9 ± 7.0	P = 0.04
Lateral septum (dorsal part)	0.86	400.3 ± 16.6	306.1 ± 39.5	P = 0.02
Globus pallidus	–0.82	360.0 ± 40.4	246. 6 ± 46.8	P = 0.04
CA1 (hippocampus)	–1.82	47.5 ± 2.5	42.8 ± 2.3	ns
CA3 (hippocampus)	–1.82	48.7 ± 2.9	40.3 ± 3.9	ns
Dentate gyrus (hippocampus)	–1.82	38.7 ± 2.4	38.2 ± 3.9	ns
Subthalamic nucleus	–2.06	234.4 ± 25.0	143.9 ± 19.8	P = 0.008
Substantia nigra compacta	–2.92	553.8 ± 31.8	427.4 ± 52.7	P = 0.03
Red nucleus	–3.80	266.8 ± 21.4	249.3 ± 27.1	ns
Pontine gray	–4.16	1750.6 ± 105.8	1618.0 ± 146.7	ns
Pedunculopontine nucleus	–4.48	126.5 ± 9.0	105.6 ± 14.7	ns
Principal sensory nucleus V	–4.96	674.0 ± 29.9	546.6 ± 64.0	P = 0.04
Interposed cerebellar nucleus	–6.12	422.9 ± 33.2	337.6 ± 40.8	ns

ns, statistically not significant.

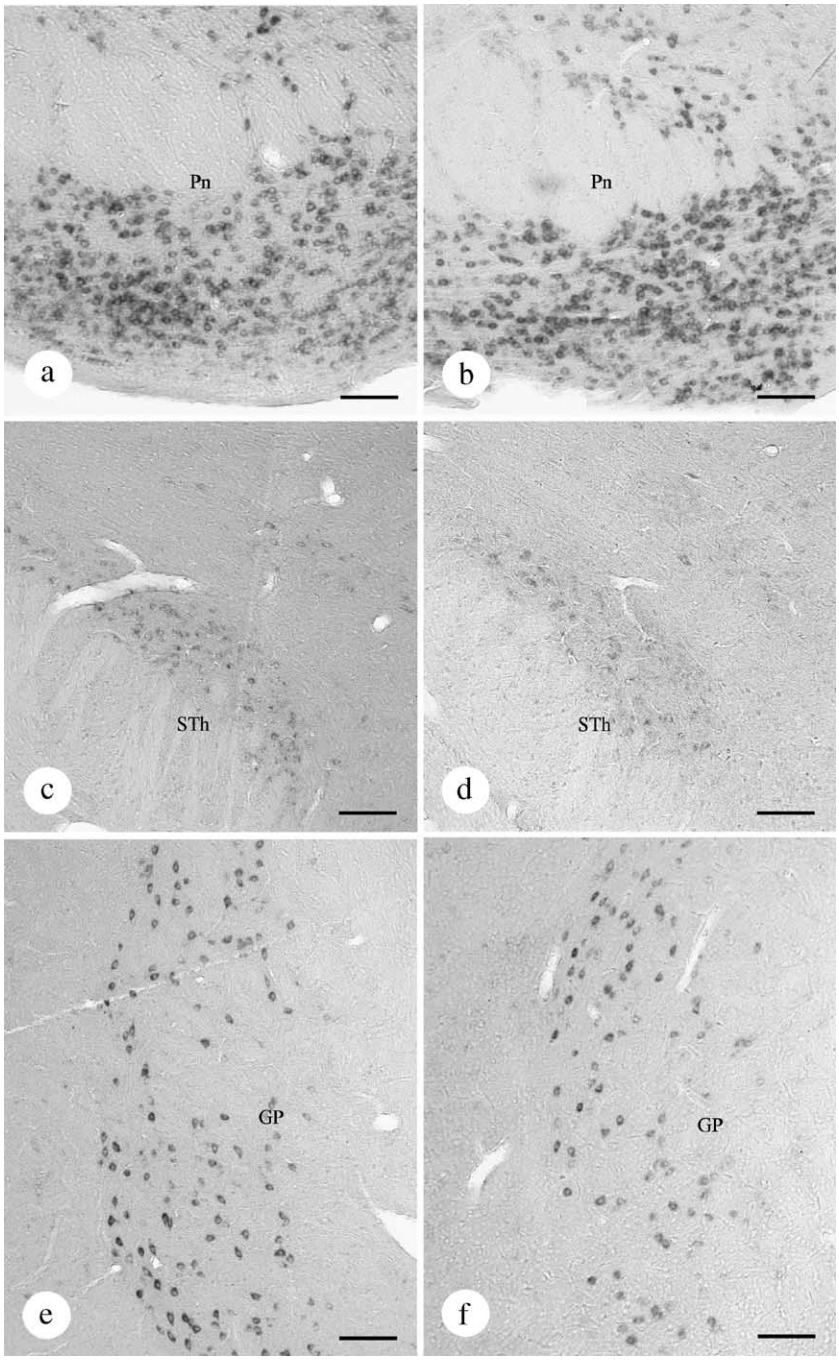

Fig. 2 – Comparison of in situ hybridization of CRF1 mRNA in three brain structures, between WT (left panels) and CRF-OE (right panels) mice. The number of positive neurons in the pontine gray does not differ (a, b), but in the subthalamic nucleus and globus pallidus, it is much lower in CRF-OE (e, f) than in WT (c, d) mice. GP: globus pallidus; Pn: pontine gray; STh: subthalamic nucleus. Scale bar: 50 μm.

the CRF-OE mice, markedly lower numbers of CRF1 mRNA-positive cell bodies were observed in the subthalamic nucleus (STh) (–38.6%) (Figs. 2c, d) and in the GP (–31.5%) (Figs. 2e, f), whereas, moreover, lower numbers were also observed in the dorsal part of the LS (–23.5%), the SN (–22,8%), the principal sensory V nucleus (–18.9%) and the somatosensory cortex (–18.4%). Detailed data are given in Table 1.

2.3. *CRF2 mRNA distribution in WT and CRF-OE mouse brain*

Our quantitative analysis shows that no differences exist between WT and CRF-OE mice in the number of CRF2 mRNA-positive neurons in the medial nucleus of the amygdala, the intermediate part of the LS (Figs. 3a, b), the posterodorsal region of the BNST, the VMH and the median raphe nucleus.

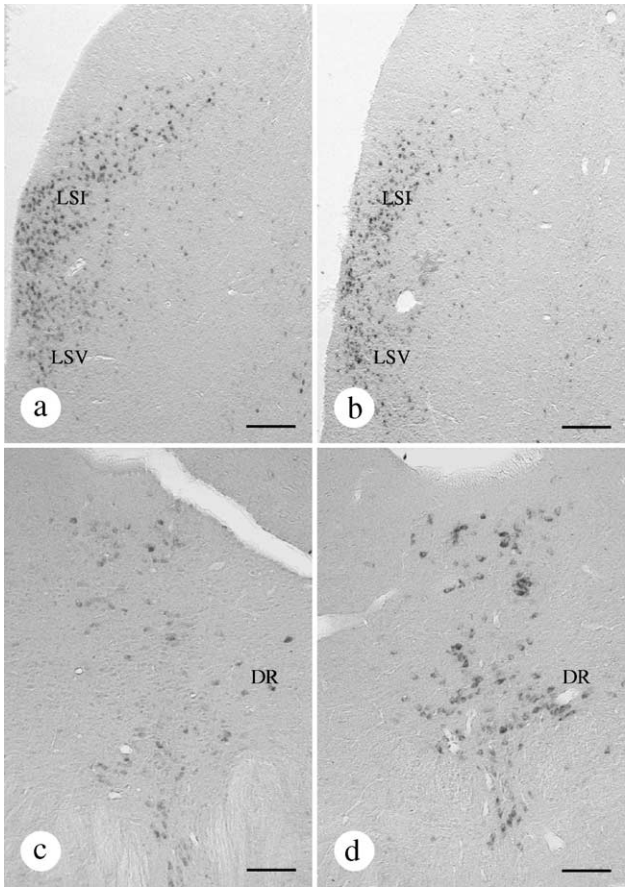

Fig. 3 – Comparison of in situ hybridization of CRF2 mRNA in two brain structures, between WT (left panels) and CRF-OE (right panels) mice. The number of positive neurons in the lateral septum does not differ (a, b), but in the dorsal raphe nucleus, it is much higher in CRF-OE (d) than in WT (c) mice. DR: dorsal raphe nucleus; LSI: intermediate part of lateral septum; LSV: ventral part of lateral septum. Scale bar: 50 μm.

However, the number of CRF2 mRNA-positive neurons in the DR (Figs. 3c, d) in the CRF-OE mouse brain is clearly higher (+32.2%) than that in the WT. Detailed data are given in Table 2.

3. Discussion

3.1. *General considerations*

In the present study, we investigated the possible involvement of CRF in the regulation of CRF1 and CRF2 receptors in the mouse brain. This has been done by testing the hypothesis that CRF controls, differentially as to receptor type and brain structure, the expressions of CRF1 mRNA and CRF2 mRNA, in a mouse strain with chronic CRF over-expression in the central nervous system (Dirks et al., 2002a,b; Groenink et al., 2002). In addition, as this CRF-OE mouse shows a number of physiological and behavioral aberrations from WT mice, our data provide a basis for the further elucidation of the functional significance of these two CRF receptor types and the brain areas that express them. These two aspects of our studies will be discussed below.

3.2. *CRF over-expression leads to differential expression of CRF1 and CRF2 receptors*

Our immunocytochemical study shows strong hybridization signals with probes for CRF1 mRNA and CRF2 mRNA in various brain structures of WT and CRF-OE mice. The absence of background reaction and of any positive signal with control sense probes indicates that the signals specifically reveal the presence of CRF1 and CRF2 mRNAs in neuronal cell bodies. The anatomical analysis of the WT brain demonstrates that the two receptor mRNAs have non-overlapping patterns and that these patterns are essentially the same as described previously for the brain of the same mouse C57BL/6 strain (Van Pett et al., 2000). The distinctly different distributions of the two mRNAs suggest that the CRF1 and CRF2 receptors have different functions.

The same anatomical distributions of the two receptor mRNAs as occur in WT mice were found in all CRF-OE mice. Therefore, we conclude that chronically elevated CRF does not induce CRF receptor expression in brain areas that normally (in WT) do not express these receptors and, similarly, does not inhibit these expressions in areas that normally do express these receptors. In our CRF-OE mice, CRF is expressed in brain structures that do not express CRF in normal mice (Dirks et al., 2002a). Apparently, these 'novel CRF-expressing areas' are not associated with 'novel CRF receptor-expressing areas' in the CRF-OE mouse brain. Assuming that these novel areas release CRF, it remains to be established if this CRF acts on targets also present in WT mice or on targets present in the mutant only.

As to the lack of a visible effect of CRF over-expression on CRF receptor expression in the PVN, this result is in line with the observation that restraint stress-induced CRF mRNA expression in the mouse PVN was not associated with CRF1

Table 2 – Number of neurons in brain structures with their Bregma coordinates according to Paxinos and Franklin (2001), expressing corticotropin-releasing factor receptor 2 (CRF2) mRNA in WT and CRF-OE mouse brains ($n = 7$)

Brain area	Bregma (mm)	WT	CRF-OE	*t* test
Lateral septal nucleus (intermediate part)	0.86	784.8 ± 59.7	744.2 ± 65.9	ns
Bed nucleus of the stria terminalis (posterodorsal part)	–0.46	187.8 ± 42.0	223.7 ± 30.2	ns
Medial nucleus of the amygdala	–1.22	110.1 ± 7.0	98.7 ± 7.4	ns
Ventromedial nucleus of the hypothalamus	–1.22	96.0 ± 8.9	100.0 ± 7.5	ns
Dorsal raphe	–4.60	73.9 ± 3.0	97.7 ± 5.4	$P = 0.001$
Median raphe	–4.60	49.6 ± 7.5	53.8 ± 5.0	ns

ns, statistically not significant.

expression in this nucleus (Imaki et al., 2003). In rat, in contrast, the same stressor as well as exogenous CRF administration induces CRF1 mRNA in the PVN (Imaki et al., 1996, 2003; Mansi et al., 1996). Therefore, our data support the notion that CRF-dependent regulatory mechanisms controlling CRF receptor expression in the PVN differ between mouse and rat.

Although our finding that the distribution patterns of CRF1 mRNA and CRF2 mRNA do not differ between WT and CRF-OE mice might suggest that CRF excess does not affect the expression of the receptors, the detailed quantitation of the numbers of neuronal cell body profile positive with the respective receptor mRNA probes shows a different picture. Our analyses clearly demonstrate that the degree of expression of both receptor mRNAs is substantially different when individual brain structures are considered. In a number of structures, no differences in the number of receptor mRNA-expressing neurons were seen. However, the fact that, compared to WT mice, CRF-OE mice exhibit a lower number of CRF1 mRNA-expressing neurons in the dorsal part of the LS, the GP, SN pars compacta, STh, primary somatosensory cortex and principal sensory nucleus V whereas a higher number of CRF2 mRNA-expressing neurons were found in the DR strongly indicates that a chronically elevated CRF level is associated with down-regulation of CRF1 mRNA and up-regulation of CRF2 mRNA in a brain-area-specific way. This conclusion confirms and extends the assumption of Brunson et al. (2002) in rat that CRF may regulate CRF1 and CRF2 in a differential manner.

3.3. *Possible regulatory mechanisms of CRF1 and CRF2 receptor expression*

The question emerges as to the precise relationship between CRF over-expression and changed CRF1 and CRF2 expressions. Obviously, the primary cause may be an elevated release and binding of CRF to these receptors, but the effect of CRF may be more indirect, e.g. via CRF-induced release of other factors known to be involved in stress-related neuronal activities, such as glucocorticoids and other CRF-related peptides. This idea receives support from studies on CRF receptor mRNA expression in rat, where chronic unpredictable stress decreases CRF1 mRNA in the frontal cortex and increases CRF1 mRNA in the hippocampus (Iredale et al., 1996), chronic corticosterone administration decreases CRF1 mRNA in the PVN and the BLA (Makino et al., 1995, 1997) and increases CRF2 mRNA in the VMH (Makino et al., 1997, 1998), and subchronic exposure to $GABA_A$-benzodiazepines receptor agonist decreases CRF1 mRNA in the BLA but increases CRF2 mRNA in the LS (Skelton et al., 2000a). These data reveal that not only CRF but also other stress-related factors may control CRF1 and CRF2 expression, but also indicate that the same factors can have opposite effects on different brain structures and can regulate the two receptor types in opposite ways. The present study is the first to show that such opposite regulations, either by CRF and/or by such other factors, can occur in the mouse brain. In this respect, it is noteworthy that the CRF-OE mouse shows an elevated basal plasma corticosterone level (Groenink et al., 2002) and down-regulation of Ucn1 in the E–WN (Kozicz et al., 2004). Ucn1 is a CRF-related peptide, which also acts through CRF1 and CRF2, and has been implicated in the regulation of the stress response and more specifically in the control of CRF-regulated adaptive processes (Latchman, 2002; Skelton et al., 2000b; Oki and Sasano, 2004). Below, we will look in some detail how CRF and other stress-regulating factors might control some of the brain structures in CRF-OE that express CRF receptor mRNA differently from WT mice.

As to the dorsal part of the LS, the down-regulation of CRF1 mRNA may be under the control of CRF-producing neurons in the hypothalamus, which shows a strong up-regulation of CRF in CRF-OE mice (Dirks et al., 2002a). Possibly, the projection from this nucleus to the LS is involved in the control of various types of behavior including stress-related anxiety (Sakanaka et al., 1988). Similarly, the down-regulation of CRF1 mRNA observed in the SN might be caused by CRF neurons located in the CeA and the BNST, which also show a clear up-regulation of CRF in CRF-OE mice (Dirks et al., 2002a). The latter two brain areas innervate dopaminergic neurons in the substantia nigra and therefore are likely to modulate dopaminergic activity in the striatum (Gray, 1993). Furthermore, an increased expression of CRF2 mRNA in the DR may not (only) be caused by over-expressed CRF but (also) by decreased expression of Ucn1 (Kozicz et al., 2004). In CRF-OE mice, such a decrease has been reported in the E–WN, which has major projections to the DR nucleus (Chung et al., 1987; Vaughan et al., 1995; Bittencourt and Sawchenko, 2000; Bittencourt et al., 1999; Weitemier et al., 2005). The E–WN Ucn1 acts through both CRF1 and CRF2 receptors but has a strong preference for CRF2 (Potter et al., 1994; Turnbull and Rivier, 1997; Reul and Holsboer, 2002).

3.4. *Possible functional significance of changed receptor expressions*

Clearly, the control of physiological and cognitive functions does not only involve changes in CRF release but also plastic changes in the expressions of CRF1 and CRF2. This makes the CRF-OE mouse a suitable model to obtain information about the role of CRF receptors in these physiological and cognitive functions, namely, in relating brain-site-specific changes in CRFR content to specific physiological or cognitive aberrations. Below, we will apply this approach to a number of physiologically rather well-defined brain structures.

Among the brain areas with a lower number of CRF1 mRNA-positive neurons, the strongest effect of CRF over-expression was observed in the STh, the GP and the SN, all belonging to the basal nuclei system. These ganglia are considered as important nodes in cortico-subcortical networks involved in the processing of information in motor, cognitive and limbic domains (Tisch et al., 2004). Indeed, CRF-OE mice reveal reduced and non-habituating startle reactivity as well as impairment of prepulse inhibition (Dirks et al., 2002b), symptoms of impaired information processing. Therefore, the current data suggest that CRF1 in basal nuclei may be important for adequate information processing.

The only difference in CRF2 expression observed in CRF-OE mice when compared to WT is a remarkably higher number of mRNA-positive cell body profiles in the DR nucleus that also exhibits the vast majority of serotoninergic neurons in mammals (Dahlstrom and Fuxe, 1964; Abrams et al., 2004). The prominent participation of serotonin is generally

acknowledged in stress-induced arousal as occurs during anxiety and depression (for reviews, see e.g. Graeff et al., 1996, 1997; Carrasco and Van de Kar, 2003). Microinjection of CRF into the dorsal raphe nucleus alters raphe neuronal activity in vivo (Kirby et al., 2000) and in vitro (Kirby et al., 2000; Lowry et al., 2000; Price et al., 2002), and the tone of the raphe–serotonin system is regulated in a dynamic manner through CRF-R2 activation (Pernar et al., 2004). Therefore, our result of an increased expression of DR CRF2 in CRF-OE mice may contribute to the identification of the neuronal networks involved in stress-related diseases such as anxiety and major depression.

4. Experimental procedures

4.1. *Animals*

CRF-OE mice were generated as previously described (Dirks et al., 2002a). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (0.6 kb fragment; (Thompson et al., 1987), which was inserted at the *Xho*I restriction site into an 8.2 kb *Eco*RI genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence (Aigner et al., 1995). The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons (Morris and Grosveld, 1989; Vidal et al., 1990; Luthi et al., 1997). The Thy-1 CRF gene was prepared for microinjection by isolating a 9.0 kb *Not*I fragment containing the fusion gene, which was purified from a conventional agarose gel by electroelution. The fragment was microinjected into fertilized eggs (C57BL/6J), and the injected cells were transplanted into pseudopregnant foster mothers. To identify transgenic founder animals, tail DNA from offspring was screened by standard Southern dot-blot analysis using the 0.6 kb CRF cDNA fragment as a probe. These procedures yielded three transgenic founder animals, which gave rise to three independent lines of transgenic animals, one of which (CRF-OE 2122 line) was bred further at the local breeding facilities (Central Laboratory Animal Institute, Utrecht University, The Netherlands) and used for the present study. Breeding consisted of mating between transgenic male and C57BL/6J female mice. Tail DNA from offspring, extracted with the High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany), was screened using PCR with transgene-specific primers. The forward primers were specific for rat CRF and the reverse primers originated from the Thy-1 promoter, thus excluding the possibility that the endogenous CRF and Thy-1 genes were amplified.

Adult male transgenic CRF-OE mice (n = 7) were used in all experiments. Adult male littermate WT mice served as controls (n = 7). Mice were housed 2–3 per cage, had free access to food and water and were adapted to housing conditions 14 days prior to perfusion for histology. All procedures were approved by the ethical committee for animal experimentation of the Faculties of Pharmaceutical Sciences, Biology and Chemistry, Utrecht University (Dec-GNK-FSB), according to the Dutch law for animal experimentation and the Declaration of Helsinki.

4.2. *Tissue processing*

Animals were deeply anesthetized with nembutal® (60 mg/ml sodium pentobarbital, Sanofi Santé B.V., Maassluis, The Netherlands; 0.1 ml/mouse intraperitoneally) and perfused transcardially with sterile saline followed by freshly prepared 4% paraformaldehyde in RNAse-free 0.1 M sodium phosphate buffer (pH 7.4; 100 ml/mouse). After perfusion, animals were decapitated, and brains were removed and postfixed in 4% paraformaldehyde for 16 h at 4 °C and subsequently stored in autoclaved 0.1 M sodium phosphate-buffered saline (PBS) at 4 °C until use. Prior to cutting, tissues were transferred to 30% sucrose in 0.1 M PBS for 16 h at 20 °C and then frozen with dry ice. Of each brain, 30-μm-thick coronal sections with 150-μm intervals were saved in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at –20 °C, until histological processing.

4.3. *In situ hybridization*

In situ hybridization for CRF1 and CRF2α mRNAs was carried out with the free-floating section method according to Jessell (http://www.cpmcnet.columbia.edu/dept/neurobeh/jessell/insitu.html), with minor modifications, as follows. Antisense and sense (control) RNA probes were generated using rat CRF1 and CRF2α cDNAs (kindly provided by Dr. W.W. Vale, San Diego, CA, USA) and labeled with DIG-11-UTP using a labeling kit from Roche Molecular Biochemicals (Basel, Switzerland). In situ hybridization steps were carried out at room temperature (20 °C) unless stated otherwise. First, sections were fixed in 0.1 M borax-buffered 4% paraformaldehyde (pH 9.5) at 4 °C for 30 min. Then, sections were rinsed four times for 7 min with 0.1 M PBS followed by preincubation in proteinase K medium (0.1 M Tris/HCl, 0.05 M EDTA, 0.1 mg proteinase K) for 10 min at 37 °C. After rinsing in autoclaved MQ water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by rinsing in 2 times concentrated (2×) standard saline citrate buffer (SSC; pH 7.0) for 5 min. Hybridization mixture (50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, Denhardt's solution, 10% dextran sulfate), together with 0.5 mg/ml tRNA and the mRNA digoxigenin (DIG) probe (ca. 2.5 ng/ml), was placed into a water bath at 80 °C for 5 min and then on ice for another 5 min. Sections were incubated in hybridization solution for 16 h at 60 °C, rinsed four times for 7 min with 4× SSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, 0.01 mg/ml RNAse A; pH 8.0) that had been added just before the start of incubation and stringently washed in steps with decreasing SSC concentrations (2×, 1×, 0.5×, 0.1×) for 30 min at 58 °C. The alkaline phosphatase method with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) as substrate was used for the detection of the DIG label. Briefly, after rinsing four times for 5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5), sections were preincubated in buffer A containing 0.5% blocking agent (Roche Molecular Biochemicals) for 60 min followed by 3 h of incubation with sheep anti-DIG-AP (Roche Molecular Biochemicals; 1:5000) in buffer A containing 0.5% blocking agent. Subsequently, sections were rinsed four times for 5 min in buffer A followed by two times of 5 min of rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M $MgCl_2$; pH 9.5). After 16 h of incubation in NBT/BCIP medium (10 ml buffer B, 2.4 mg levamisole, 175 μl NBT/BCIP mixture; Roche Molecular Biochemicals) in a light-tight box, the reaction was stopped by placing the sections in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0). After rinsing twice for 5 min, sections were mounted on gelatin-coated glasses, dried for 16 h at 37 °C, rinsed in distilled water, dehydrated, cleared in xylene and coverslipped with Entellan. Sections of WT and CRF-OE mice brains were processed for in situ hybridization in parallel for each CRF receptor mRNA.

4.4. *Quantitative analyses*

To obtain quantitative information about the presence of receptor (CRF1 and CRF2) mRNAs in various brain structures, the numbers of neuronal cell body profiles in in situ hybridized sections were counted by direct microscopic examination, by an observer who was neuroanatomically experienced but unaware of the scientific aim of the study and of the key of the encoded slides. Counts were carried out using a Zeiss Axiovert 35 M microscope (Carl Zeiss AG, Germany) and Neurolucida software (MicroBrightfield, Williston, VT, USA). All cells that were visible, i.e., contained receptor mRNA,

were counted. Moreover, only cells with a clearly visible nucleus were counted. Per receptor type and experimental group (WT, CRF-OE), brains of 7 animals were studied. Per brain, cell body profiles with a positive hybridization signal for a receptor mRNA were counted in the two most central sections present of a brain structure, according to the coordinates given in the atlas of Paxinos and Franklin (2001). The brain structures and their coordinates are given in Tables 1 and 2. Per structure, the counts of the two sections were averaged, and the average value obtained for each mouse was used to calculate group means. The area of measurement included the whole brain structure, except for the somatosensory cortex (layers I–IV) and the CA1, CA3 and dentate gyrus of the hippocampus, which were analyzed in a central square (0.040 mm^2 for the somatosensory cortex, 0.013 mm^2 for each hippocampal region). Data were statistically analyzed with Student's unpaired *t* test ($\alpha = 5\%$).

REFERENCES

Abrams, J.K., Johnson, P.L., Hollis, J.H., Lowry, C.A., 2004. Anatomic and functional topography of the dorsal raphe nucleus. Ann. N. Y. Acad. Sci. 1018, 46–57.

Aigner, L., Arber, S., Kapfhammer, J.P., Laux, T., Schneider, C., Botteri, F., Brenner, H.R., Caroni, P., 1995. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell 83, 269–278.

Bittencourt, J.C., Sawchenko, P.E., 2000. Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. J. Neurosci. 20, 1142–1156.

Bittencourt, J.C., Vaughan, J., Arias, C., Rissman, R.A., Vale, W.W., Sawchenko, P.E., 1999. Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors. J. Comp. Neurol. 415, 285–312.

Bonaz, B., Rivest, S., 1998. Effect of a chronic stress on CRF neuronal activity and expression of its type 1 receptor in the rat brain. Am. J. Physiol. 275, 1438–1449.

Brunson, K.L., Grigoriadis, D.E., Lorang, M.T., Baram, T.Z., 2002. Corticotropin-releasing hormone (CRH) downregulates the function of its receptor (CRF1) and induces CRF1 expression in hippocampal and cortical regions of the immature rat brain. Exp. Neurol. 176, 75–86.

Carrasco, G.A., Van de Kar, L.D., 2003. Neuroendocrine pharmacology of stress. Eur. J. Pharmacol. 463, 235–272.

Chalmers, D.T., Lovenberg, T.W., De Souza, E.B., 1995. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J. Neurosci. 15, 6340–6350.

Chung, R.Y., Mason, P., Strassman, A., Maciewicz, R., 1987. Edinger–Westphal nucleus: cells that project to spinal cord contain corticotropin-releasing factor. Neurosci. Lett. 83, 13–19.

Dahlstrom, A., Fuxe, K., 1964. Localization of monoamines in the lower brain stem. Experientia 20, 398–399.

Dirks, A., Groenink, L., Bouwknecht, J.A., Hijzen, T.H., van der Gugten, J., Ronken, E., Verbeek, J.S., Veening, J.G., Dederen, P.J., Korosi, A., Schoolderman, L.F., Roubos, E.W., Olivier, B., 2002a. Overexpression of corticotropin-releasing hormone in transgenic mice and chronic stress-like autonomic and physiological alterations. Eur. J. Neurosci. 16, 1751–1760.

Dirks, A., Groenink, L., Schipholt, M.I., van der Gugten, J., Hijzen, T.H., Geyer, M.A., Olivier, B., 2002b. Reduced startle reactivity and plasticity in transgenic mice overexpressing corticotropin-releasing hormone. Biol. Psychiatry 51, 583–590.

Fisher, L.A., 1989. Corticotropin-releasing factor: endocrine and autonomic integration of responses to stress. Trends Pharmacol. Sci. 10, 189–193.

Graeff, F.G., Guimaraes, F.S., De Andrade, T.G., Deakin, J.F., 1996. Role of 5-HT in stress, anxiety, and depression. Pharmacol. Biochem. Behav. 54, 129–141.

Graeff, F.G., Viana, M.B., Mora, P.O., 1997. Dual role of 5-HT in defense and anxiety. Neurosci. Biobehav. Rev. 21, 791–799.

Gray, T.S., 1993. Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress. Ann. N. Y. Acad. Sci. 697, 53–60.

Groenink, L., Dirks, A., Verdouw, P.M., Schipholt, M., Veening, J.G., van der Gugten, J., Olivier, B., 2002. HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. Biol. Psychiatry 51, 875–881.

Groenink, L., Pattij, T., De Jongh, R., van der Gugten, J., Oosting, R.S., Dirks, A., Olivier, B., 2003. 5-HT1A receptor knockout mice and mice overexpressing corticotropin-releasing hormone in models of anxiety. Eur. J. Pharmacol. 463, 185–197.

Holsboer, F., Lauer, C.J., Schreiber, W., Krieg, J.C., 1995. Altered hypothalamic–pituitary–adrenocortical regulation in healthy subjects at high familial risk for affective disorders. Neuroendocrinology 62, 340–347.

Imaki, T., Naruse, M., Harada, S., Chikada, N., Imaki, J., Onodera, H., Demura, H., Vale, W., 1996. Corticotropin-releasing factor up-regulates its own receptor mRNA in the paraventricular nucleus of the hypothalamus. Mol. Brain Res. 38, 166–170.

Imaki, T., Katsumata, H., Miyata, M., Naruse, M., Imaki, J., Minami, S., 2001. Expression of corticotropin-releasing hormone type 1 receptor in paraventricular nucleus after acute stress. Neuroendocrinology 73, 293–301.

Imaki, T., Katsumata, H., Konishi, S.I., Kasagi, Y., Minami, S., 2003. Corticotropin-releasing factor type-1 receptor mRNA is not induced in mouse hypothalamus by either stress or osmotic stimulation. J. Neuroendocrinol. 15, 916–924.

Iredale, P.A., Terwilliger, R., Widnell, K.L., Nestler, E.J., Duman, R.S., 1996. Differential regulation of corticotropin-releasing factor1 receptor expression by stress and agonist treatments in brain and cultured cells. Mol. Pharmacol. 50, 1103–1110.

Kirby, L.G., Rice, K.C., Valentino, R.J., 2000. Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. Neuropsychopharmacology 22, 148–162.

Kozicz, T., Korosi, A., Korsman, C., Tilburg-Ouwens, D., Groenink, L., Veening, J., van der Gugten, J., Roubos, E., Olivier, B., 2004. Urocortin expression in the Edinger–Westphal nucleus is down-regulated in transgenic mice over-expressing neuronal corticotropin-releasing factor. Neuroscience 123, 589–594.

Latchman, D.S., 2002. Urocortin. Int. J. Biochem. Cell Biol. 34, 907–910.

Lowry, C.A., Rodda, J.E., Lightman, S.L., Ingram, C.D., 2000. Corticotropin-releasing factor increases in vitro firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for activation of a topographically organized mesolimbocortical serotonergic system. J. Neurosci. 20, 7728–7736.

Luo, X., Kiss, A., Makara, G., Lolait, S.J., Aguilera, G., 1994. Stress-specific regulation of corticotropin releasing hormone receptor expression in the paraventricular and supraoptic nuclei of the hypothalamus in the rat. J. Neuroendocrinol. 6, 689–696.

Luthi, A., Van der, P.H., Botteri, F.M., Mansuy, I.M., Meins, M., Frey, U., Sansig, G., Portet, C., Schmutz, M., Schroder, M., Nitsch, C., Laurent, J.P., Monard, D., 1997. Endogenous serine protease inhibitor modulates epileptic activity and hippocampal long-term potentiation. J. Neurosci. 17, 4688–4699.

Makino, S., Schulkin, J., Smith, M.A., Pacak, K., Palkovits, M., Gold, P.W., 1995. Regulation of corticotropin-releasing hormone receptor messenger ribonucleic acid in the rat brain and

pituitary by glucocorticoids and stress. Endocrinology 136, 4517–4525.
Makino, S., Takemura, T., Asaba, K., Nishiyama, M., Takao, T., Hashimoto, K., 1997. Differential regulation of type-1 and type-2alpha corticotropin-releasing hormone receptor mRNA in the hypothalamic paraventricular nucleus of the rat. Mol. Brain Res. 47, 170–176.
Makino, S., Nishiyama, M., Asaba, K., Gold, P.W., Hashimoto, K., 1998. Altered expression of type 2 CRH receptor mRNA in the VMH by glucocorticoids and starvation. Am. J. Physiol. 275, 1138–1145.
Mansi, J.A., Rivest, S., Drolet, G., 1996. Regulation of corticotropin-releasing factor type 1 (CRF1) receptor messenger ribonucleic acid in the paraventricular nucleus of rat hypothalamus by exogenous CRF. Endocrinology 137, 4619–4629.
Morris, R., Grosveld, F., 1989. Expression of Thy-1 in the nervous system of the rat and mouse. Immunol. Ser. 45, 121–148.
Nemeroff, C.B., 1996. The corticotropin-releasing factor (CRF) hypothesis of depression: new findings and new directions. Mol. Psychiatry 1, 336–342.
Oki, Y., Sasano, H., 2004. Localization and physiological roles of urocortin. Peptides 25, 1745–1749.
Paxinos, G., Franklin, F.B.J., 2001. The Mouse Brain in Stereotaxic Coordinates. Academic Press.
Pernar, L., Curtis, A.L., Vale, W.W., Rivier, J.E., Valentino, R.J., 2004. Selective activation of corticotropin-releasing factor-2 receptors on neurochemically identified neurons in the rat dorsal raphe nucleus reveals dual actions. J. Neurosci. 24, 1305–1311.
Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P.E., Vale, W., 1994. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc. Natl. Acad. Sci. U. S. A. 91, 8777–8781.
Price, M.L., Kirby, L.G., Valentino, R.J., Lucki, I., 2002. Evidence for corticotropin-releasing factor regulation of serotonin in the lateral septum during acute swim stress: adaptation produced by repeated swimming. Psychopharmacology (Berlin) 162, 406–414.
Reul, J.M., Holsboer, F., 2002. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr. Opin. Pharmacol. 2, 23–33.
Rivest, S., Laflamme, N., Nappi, R.E., 1995. Immune challenge and immobilization stress induce transcription of the gene encoding the CRF receptor in selective nuclei of the rat hypothalamus. J. Neurosci. 15, 2680–2695.
Sakanaka, M., Magari, S., Shibasaki, T., Lederis, K., 1988. Corticotropin releasing factor-containing afferents to the lateral septum of the rat brain. J. Comp. Neurol. 270, 404–407.
Skelton, K.H., Nemeroff, C.B., Knight, D.L., Owens, M.J., 2000a. Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. J. Neurosci. 20, 1240–1248.
Skelton, K.H., Owens, M.J., Nemeroff, C.B., 2000b. The neurobiology of urocortin. Regul. Pept. 93, 85–92.
Swanson, L.W., Sawchenko, P.E., Rivier, J., Vale, W., 1983. Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36, 165–186.
Thompson, R.C., Seasholtz, A.F., Herbert, E., 1987. Rat corticotropin-releasing hormone gene: sequence and tissue-specific expression. Mol. Endocrinol. 1, 363–370.
Tisch, S., Silberstein, P., Limousin-Dowsey, P., Jahanshahi, M., 2004. The basal ganglia: anatomy, physiology, and pharmacology. Psychiatry Clin. North Am. 27, 757–799.
Turnbull, A.V., Rivier, C., 1997. Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. Proc. Soc. Exp. Biol. Med. 215, 1–10.
Vale, W., Spiess, J., Rivier, C., Rivier, J., 1981. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213, 1394–1397.
Valentino, R.J., Page, M., Van Bockstaele, E., Aston-Jones, G., 1992. Corticotropin-releasing factor innervation of the locus coeruleus region: distribution of fibers and sources of input. Neuroscience 48, 689–705.
Van Pett, K., Viau, V., Bittencourt, J.C., Chan, R.K., Li, H.Y., Arias, C., Prins, G.S., Perrin, M., Vale, W., Sawchenko, P.E., 2000. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J. Comp. Neurol. 428, 191–212.
Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378, 287–292.
Vidal, M., Morris, R., Grosveld, F., Spanopoulou, E., 1990. Tissue-specific control elements of the Thy-1 gene. EMBO J. 9, 833–840.
Weitemier, A.Z., Tsivkovskaia, N.O., Ryabinin, A.E., 2005. Urocortin 1 distribution in mouse brain is strain-dependent. Neuroscience 132, 729–740.