

Targeted mutations of the corticotropin-releasing factor system: effects on physiology and behavior

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Summary Genetic modifications of the genes that encode proteins integral to the corticotropin-releasing factor (CRF) system have been employed in the creation of mutant mice that serve as tools for studying the role of this neuropeptide in regulated and dysregulated behaviors and physiology. Overexpression of the CRF peptide and CRF binding protein as well as deletion of the peptide, binding protein, and both known receptors has been achieved and these mouse models have been characterized for anatomical, neuroendocrine, and behavioral sequelae. The profile of results, consistent with current knowledge of CRF function from more traditional assays, indicates that enhancement of CRF function is associated with an activation of the hypothalamic-pituitary-adrenal axis, an anxious phenotype, alterations in cognitive performance and reductions in feeding. In general, blockade of CRF function produces the opposite effects. Genetic mouse models allow further analysis of specific elements in the CRF circuitry for which more traditional tools have not existed. These animal models are valuable for increasing our understanding of the underlying pathology associated with a variety of psychiatric and neuroendocrine disorders and for the development and testing of novel treatment agents. © 2002 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Currently, two different receptors are known to mediate the biological actions of CRF, CRFR1, and CRFR2 (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993; Lovenberg et al., 1995b; Perrin et al., 1995). Two splice variants of CRFR2 have been described, designated as CRFR2 α and CRFR2 β , and in humans, a third splice variant (CRFR2 γ) has also been identified (Lovenberg et al., 1995a; Kostich et al., 1998). CRFR1 and CRFR2 possess distinct pharmacological profiles and distribution patterns in brain and peripheral rat tissues (Chalmers et al., 1995; Primus et al., 1997; see Fig. 1). CRFR1 is mainly found in the cortex, cerebellum, amygdala, and pituitary

gland whereas CRFR2 is predominantly expressed in the lateral septum and hypothalamic nuclei. More recently, examination of brain CRFR1 and CRFR2 in nonhuman primates revealed similar receptor distribution patterns with the exception of higher densities of CRFR2 in the neocortex, hippocampus, and amygdala as compared to the rat brain (Sanchez et al., 1999). Finally, CRFR1-like immunoreactivity, CRFR1 and CRFR2 mRNA distribution patterns in the mouse brain have been shown to largely overlap with those observed in rat studies (Chen et al., 2000; van Pett et al., 2000).

HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

Numerous studies indicate a major role for hypothalamic CRF in the regulation of basal and stress-induced activation of the hypothalamus-pituitary-adrenal (HPA) axis (for a review see Muglia et al., 2001; Venihaki and Majzoub, this issue). CRF stimulates the release of ACTH

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CRF receptor mRNA distributions in rat brain

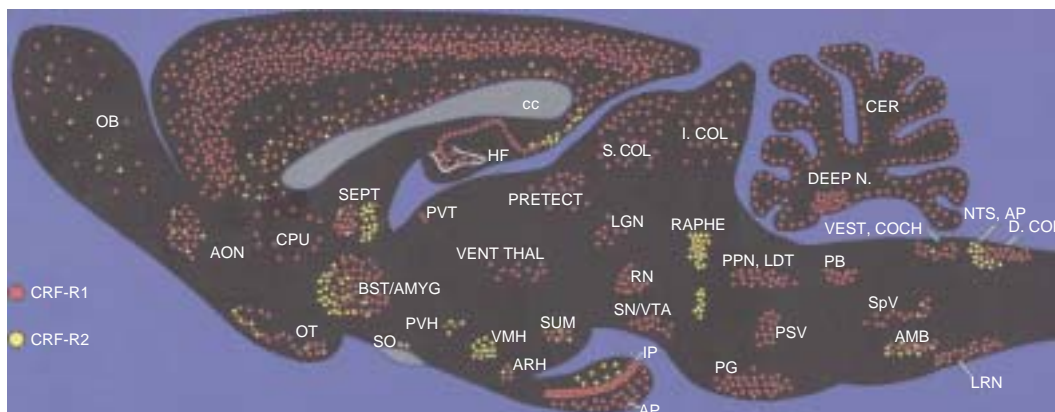


Fig. 1 CRF receptor distributions in rodent brain. Schematic drawing of sagittal section through the rat brain shows the distribution and relative density of mRNA's for CRFR1 and CRFR2. The CRFR2 transcript shows a more restricted distribution that is largely non-overlapping with that of CRFR1. Modified from van Pett et al., 2000. Abbreviations: AMB nucleus ambiguus; AMYG amygdala; AON anterior olfactory nucleus; AP anterior lobe, pituitary gland, area postrema; ARH arcuate nucleus (hypothalamus); BST bed nucleus of the stria terminalis; cc corpus callosum; CER cerebellum; COCH cochlear nuclei; CPU caudoputamen; D.COL dorsal column nuclei; DEEP N. deep nuclei (cerebellum); HF hippocampal formation; I. COL. inferior colliculus; IP intermediate lobe, pituitary gland; LDT laterodorsal tegmental nucleus; LGN lateral geniculate nucleus; LRN lateral reticular nucleus; NTS nucleus of the solitary tract; OB olfactory bulb; OT olfactory tuberle; PB parabrachial nucleus; PG pontine gray; PPN pedunculo-pontine nucleus; PRETECT pretecal region; PSV principal sensory nucleus of the trigeminal nerve; PVH paraventricular nucleus (hypothalamus); PVT paraventricular nucleus (thalamus); RN red nucleus; SEPT septal region; S. COL. superior colliculus; SN substantia nigra; SO supraoptic nucleus; SpV spinal trigeminal nucleus; SUM supramammillary nucleus; VENT THAL ventral thalamus; VEST vestibular nuclei; VMH ventromedial nucleus (hypothalamus); VTA ventral tegmental area.

from the corticotrope cells of the anterior pituitary gland, which in turn stimulates secretion of glucocorticoids, mainly corticosterone in rodents and cortisol in humans, from the cortex region of the adrenal gland. The study of the specific role for each element of the HPA axis during basal and stressful conditions has recently been aided by the generation of genetically engineered mice with targeted mutations of the CRF system.

The effect of increased CRF production was initially examined in a transgenic mouse model with brain CRF overabundance (CRF TG mice). Compared to wild-type (WT) mice, CRF TG mice displayed elevated plasma levels of ACTH and corticosterone but a preserved circadian rhythm of these hormones. Moreover, as a consequence of increased levels of corticosterone, the CRF TG mice developed physical changes, such as muscle atrophy, truncal obesity, thin skin, hair loss, and buffalo hump similar to those observed in Cushing's syndrome patients (Stenzel-Poore et al., 1992). Despite increased plasma ACTH and corticosterone levels, CRF TG mice showed attenuated HPA responses to stressful stimuli. In contrast to WT mice, CRF TG mice displayed minimal increase in plasma levels of ACTH and corticosterone, following exposure to a 10-min restraint stress (Coste et al., 2001). Lifelong enhanced negative feedback mechanisms resulting from elevated plasma corticosterone levels may underlie HPA endocrine responses observed in CRF TG mice. Similarly, impaired plasma cortisol responses to stress

have been observed in Cushing's patients. However, the increased plasma cortisol levels detected in these patients following administration of CRF or ACTH suggest greater negative feedback signals by elevated circulating cortisol at the level of the hypothalamus than healthy controls (Aron et al., 1987). Moreover, hypothalamic feedback mechanisms may underlie the normal CRF levels detected in the paraventricular nucleus (PVN) of CRF TG mice in the presence of CRF overexpression throughout the brain (Stenzel-Poore et al., 1992).

Recently, an additional CRF TG mouse model was created that employed a Thy-1 regulatory sequence to drive transgene expression in postnatal and adult neurons (Groenink et al., 2002). These CRH-OE₂₁₂₂ mice showed elevated basal plasma corticosterone concentrations, hypertrophy of the adrenal gland, and dexamethasone non-suppression but had no Cushing-like phenotype and showed a normal corticosterone response in reaction to stress.

In contrast to the endocrine phenotype observed in CRF TG mice, CRF-deficient mice (CRF KO) showed almost undetectable nadir plasma levels of corticosterone and adrenocortical atrophy. Pituitary ACTH immunoreactivity and plasma ACTH levels were, however, normal in these mice (Muglia et al., 1995, 2000). CRF KO mice also lacked the characteristic diurnal rise in plasma corticosterone levels; however, other circadian rhythms such as light:dark ambulatory patterns were preserved in these

mice (Muglia et al., 1997). In addition, foot-shock or mouse interleukin-1 β (mIL-1 β) induced a large increase in plasma corticosterone levels in WT mice whereas CRF KO mice showed no corticosterone response to the foot-shock and only a very modest increase of the glucocorticoid following treatment with mIL-1 β (Dunn and Swiergiel, 1999). Accordingly, treatment of rats with antisense oligonucleotides (AS ON) against CRF decreased plasma ACTH and corticosterone levels (Skutella et al., 1994a).

HPA axis endocrine profiles similar to those observed in CRF KO mice were also detected in null mutant mice lacking a functional type 1 receptor for CRF (CRFR1 KO). These mutant mice showed plasma levels of ACTH comparable to those observed in WT mice but very low, almost undetectable, plasma levels of corticosterone. Moreover, CRFR1 KO adult mice displayed a marked atrophy of the zona fasciculata of the adrenal gland cortex. This anatomical deficit was demonstrated to occur later in the postnatal period, since contrary to 8-week-old CRFR1-deficient mice, no sign of atrophy was detected in adrenal cortices collected from 3-day-old mutant mice. Moreover, a crucial role for ACTH in the development of the adrenal cortex was demonstrated, since 10-day-old CRFR1 KO mice showed lower ACTH plasma levels than WT mice and treatment with the pituitary hormone during postnatal days 10–21 completely rescued the adrenal gland deficit in the mutant mice (Smith et al., 1998). In contrast, no adrenal cortex atrophy was observed in an alternative mouse model of CRFR1 deficiency, which, instead, displayed a significant reduction in the size of the adrenal medulla most probably due to the very low plasma corticosterone levels observed in these mutant mice (Timpl et al., 1998). Development of the adrenal gland medulla has, in fact, been shown to depend on intact glucocorticoid signaling (Cole et al., 1995).

Despite the normal basal plasma levels of ACTH, analysis of pituitary cell cultures revealed decreased levels of ACTH in both CRFR1 heterozygous and null mutant mice as compared to WT mice, suggesting that *in vivo* other hypothalamic factors such as vasopressin (VP) may stimulate ACTH secretion and contribute to maintain normal levels of this pituitary hormone (Timpl et al., 1998). In support of this, elevated plasma VP concentrations, increased mRNA VP expression in the PVN, and VP-like immunoreactivity in the median eminence were detected in CRFR1-deficient mice (Muller et al., 2000b). In addition, treatment with a VP receptor antagonist decreased the plasma levels of ACTH in CRFR1 KO mice but not in WT mice (Muller et al., 2000b). Similar findings were obtained using anti-VP serum (Turnbull et al., 1999). Moreover, treatment with corticosterone decreased VP plasma levels in CRFR1 KO mice to those observed in WT mice. Overall, these findings indicate that up-regulation of ACTH secretagogues, such as VP, may play an important role in

the maintenance of normal plasma ACTH levels in CRFR1-deficient mice. Moreover, normalization of VP levels in CRFR1-deficient mice following treatment with corticosterone suggests that compensatory hyperactivation of the hypothalamic vasopressinergic system may depend on the glucocorticoid deficit of these mutant mice (Muller et al., 2000b).

The role for CRFR1 in stress-induced elevation of ACTH and corticosterone plasma levels has also been examined in CRFR1-deficient mice. Exposure to both restraint and forced-swim stress produced a large increase in ACTH and corticosterone plasma levels in WT mice but was without effect in CRFR1 KO mice (Smith et al., 1998; Timpl et al., 1998). This indicates a crucial role for CRFR1 in mediating HPA endocrine responses to stressful stimuli. However, pituitary–adrenal responses independent from hypothalamic mechanisms have been demonstrated in CRFR1 KO mice. The null mutant mice showed pronounced increases in ACTH and corticosterone plasma levels following turpentine-induced local inflammation which, in contrast to WT mice, were not modified by either anti-CRF or anti-VP sera (Turnbull et al., 1999).

More recently, CRFR1 KO mice have been shown to display a significant up-regulation of ACTH receptor mRNA levels in the zona fasciculata of the adrenal cortex. The same study also demonstrated the presence of both mRNAs for CRFR1 and CRFR2 in the mouse adrenal and pituitary glands (Muller et al., 2001). However, despite the elevated ACTH mRNA receptor levels, intravenous ACTH administration failed to produce increased plasma corticosterone levels in the mutant mice and treatment with subcutaneous CRF produced increased plasma ACTH and corticosterone levels in WT mice but not in CRFR1-deficient mice. Based on these results, it was suggested that intra-adrenal CRF/CRFR1 pathways might regulate glucocorticoid secretion, independently of ACTH-pituitary function. However, the concomitant increases in ACTH and corticosterone plasma levels observed in WT mice treated with subcutaneous CRF make it difficult to rule out a role for ACTH in the corticosterone rise observed in these mice.

In contrast with CRFR1 KO mice, CRFR2 null mutant mice (CRFR2 KO) showed no sign of altered adrenal or pituitary gland morphology. Accordingly, CRFR2 KO mice display normal basal levels of ACTH and corticosterone. Circadian rhythms of corticosterone also appear preserved in these mutant mice. However, CRFR2 KO mice show higher HPA endocrine responses than WT mice in response to stressful stimuli. In particular, exposure to a 2-min restraint stress produced higher levels of plasma ACTH and corticosterone in CRFR2 KO than in WT mice. Moreover, following a 10-min restraint stress CRFR2 KO mice showed higher plasma levels of corticosterone but lower plasma levels of ACTH than WT mice

(Bale et al., 2000; Coste et al., 2000). The higher corticosterone levels observed in CRFR2 KO mice compared to WT mice exposed to a 2-min restraint stress suggest that earlier and/or increased negative feedback mechanisms by circulating corticosterone might be responsible for the lower ACTH plasma levels observed in these mutant mice than in WT mice during the 10-min restraint stress procedure. Finally, examination of corticosterone levels 90 min after exposure to a 5-min restraint stress also revealed higher hormonal levels in CRFR2 KO mice than in WT mice (Coste et al., 2000). Altogether, these results indicate an important role for CRFR2 in the regulation of HPA axis responses to stressful events. Taking into consideration the role for CRFR1 in stress-induced activation of the HPA axis (Smith et al., 1998; Timpl et al., 1998), it may be argued that during exposure to stressors, stimulation of CRFR2 might counterbalance CRFR1-mediated effects on ACTH and corticosterone release, thus, contributing to HPA axis homeostasis.

Recently, HPA endocrine phenotypes were also examined in mice deficient in both CRFR1 and CRFR2 (CRFR1/R2 KO). In particular, the simultaneous absence of functional CRFR1 and CRFR2 resulted in HPA axis features similar to those previously observed in CRFR1-deficient mice. CRFR1/R2 null mutant mice showed very low basal plasma levels of corticosterone and histological analyses revealed atrophied adrenal glands and unaltered pituitary gland anatomy. Moreover, similar to CRFR1-deficient mice, CRFR1/R2 KO mice did not differ from WT mice in basal plasma ACTH levels. However, CRFR1/R2 KO mice displayed basal plasma corticosterone levels significantly lower than those observed in CRFR1 KO mice, providing initial evidence in favor of a role for CRFR2 in corticosterone release. Exposure to restraint stress procedures of various durations revealed no stress-induced ACTH or corticosterone release in both CRFR1- and CRFR1/R2-deficient mice whereas robust hormonal increases were observed in WT mice (Bale et al., 2002). Accordingly, other authors showed deficient HPA endocrine responses in CRFR1/R2 KO mice exposed to restraint stress manipulations. Moreover, in the latter study no increases in plasma levels of ACTH and corticosterone were observed in CRFR1 KO and CRFR1/R2 KO mice treated with subcutaneous CRF or exposed to a 15-min social defeat experience (Preil et al., 2001).

The role for CRF binding protein (CRF-BP) in HPA axis regulation has also been investigated in mice with genetic disruptions of this CRF ligand site. To date, two different mouse models of CRF-BP overexpression have been generated; one with constitutively elevated levels of the mouse CRF-BP in the anterior pituitary gland (pitCRF-BP TG mice; Burrows et al., 1998) and the other with a more widespread overexpression of the rat CRF-BP not only in brain and pituitary areas but also in peripheral tissues

such as the liver, kidney, heart, lung, and adrenals (perCRF-BP TG mice; Lovejoy et al., 1998). Since the CRF-BP is found only in the brain and pituitary areas in rats and mice (Seasholtz et al., 2001), perCRF-BP TG mice showed elevated levels of circulating CRF BP that were not present in WT mice.

No alterations in basal plasma levels of ACTH and corticosterone were observed in either mouse model of CRF-BP overexpression and examination of corticosterone fluctuations in perCRF-BP transgenic mice showed a normal circadian pattern of corticosterone secretion, with hormonal levels being higher before lights off (Lovejoy et al., 1998). Evaluation of HPA axis responses to stressful stimuli revealed that, following exposure to a 30-min restraint stress, pitCRF-BP TG and WT mice displayed comparable increases in plasma ACTH and corticosterone levels. However, higher hypothalamic levels of CRF and VP were observed in pitCRF-BP TG mice as compared to WT mice (Burrows et al., 1998). Increased CRF and VP levels may have resulted from compensatory mechanisms elicited by the elevated binding and "neutralization" of CRF and may underlie the normal basal and stress-induced HPA axis responses detected in pitCRF-BP TG mice.

Hypothalamic CRF and VP levels have not yet been examined in CRF-BP-deficient mice (CRF-BP KO); however, these mutant mice have been reported to display normal nadir ACTH and corticosterone plasma levels as well as unaltered circadian rhythms of corticosterone. Moreover, following exposure to a 30-min restraint stress procedure, CRF-BP KO mice showed plasma ACTH and corticosterone increases similar to WT mice (Karolyi et al., 1999).

Targeted manipulations of specific CRF system elements (peptide, receptors, binding protein, etc.) have proven very useful to understand HPA axis biological actions during both physiological and pathological conditions. Dysregulation of the HPA axis is often observed in particular psychiatric diseases. A large portion of patients with major depression exhibits elevated plasma cortisol concentrations and is hyporesponsive to the suppressing effects of dexamethasone on ACTH, beta-endorphin, and cortisol plasma levels. Moreover, following intravenous administration of CRF, depressed patients demonstrate a blunted ACTH but normal cortisol responses as compared to healthy subjects. Hypercortisolemia, dexamethasone non-suppression, and blunted ACTH responses normalize upon clinical recovery (Arborelius et al., 1999). However, evidence in favor of an etiological role for HPA alterations in mental illness is still lacking. In this regard, mutant mice with innate alterations of the CRF system serve as useful tools to help elucidate the specific role for the HPA axis, leading to the pathogenesis of a subset of psychiatric disorders.

ANXIETY

Multiple studies indicate a crucial role for brain CRF pathways in mediating anxiety-like behaviors. Intracerebroventricular (ICV) administration of CRF increases the expression of anxiety-like behaviors in rats and mice and CRF receptor antagonists can block the anxiogenic-like effects of stressful events (Britton et al., 1986; Berridge and Dunn, 1989; Heinrichs et al., 1992; Menzaghi et al., 1994). In humans, elevated CRF levels in the cerebrospinal fluid (CSF) have been demonstrated in pathological conditions characterized by heightened anxiety, such as post-traumatic stress disorders and alcohol withdrawal (Adinoff et al., 1996; Bremner et al., 1997; Baker et al., 1999).

Consistent with CRF-induced anxiogenic-like effects, increased anxiety-like responses were observed in CRF TG mice. During the Elevated Plus-maze (EPM) test of anxiety, CRF TG mice spent less time in the open arms of an EPM apparatus as compared to WT mice. Anxiety-like behaviors elicited by CRF overexpression were effectively blocked by ICV treatment with the CRF antagonist α -helical CRF₉₋₄₁, indicating a direct role for elevated brain CRF levels in the anxiety-like profile of CRF TG mice (Stenzel-Poore et al., 1994). Anxiogenic-like effects of transgenic CRF overexpression were also demonstrated using a second animal model of anxiety-like behavior. Testing in the Light-Dark box (LD) task revealed that CRF-TG mice made fewer transitions between the two compartments of an LD apparatus than WT mice. This study also provided further support in favor of central mechanisms mediating CRF anxiogenic-like effects by showing that anxiety-like responses of both WT and CRF TG mice were not affected by adrenalectomy. In addition, it was shown that CRF-TG female mice displayed markedly diminished sexual receptivity as compared to female WT mice (Heinrichs et al., 1997b). The latter observation is supported by previous work showing an important role for CRF pathways in the regulation of sexual behavior (Sirinathsinghji et al., 1983).

In contrast to the anxiogenic-like effects of brain CRF overabundance, CRF KO mice have been shown not to differ from WT mice in multiple anxiety tasks. Likewise, exposure to different types of stressful stimuli or treatment with either CRF or mouse IL-1 β produced comparable increases in anxiety-like behaviors in both CRF KO and WT mice (Weninger et al., 1999; Dunn and Swiergiel, 1999). Moreover, treatment with CRF receptor antagonists (α -helical CRF₉₋₄₁ or CP 154, 526) similarly inhibited freezing behaviors displayed by CRF KO and WT mice immediately after foot-shock exposure (Weninger et al., 1999). This indicates that molecules other than CRF may interact with CRF receptors to mediate behavioral effects of stressful events. Thus, so far examination of anxiety-like

profiles has provided no evidence of altered behavioral responses in CRF KO mice. However, it should be mentioned that during the EPM test and a multi-compartment chamber paradigm used to assess exploratory behaviors, CRF KO mice displayed higher levels of grooming than WT mice. This genotype difference was observed in both unstressed mice and following exposure to a 30-min restraint stress procedure (Dunn and Swiergiel, 1999). Similarly, elevated levels of grooming have been recently also detected in CRFR1 KO mice during assessment of behavior in the home cage (Penalva et al., 2002) and CRFR1/R2 KO mice during the open-field (OF) test (Bale et al., 2002). In contrast, no change in this peculiar behavior was observed in CRFR2 KO mice (Bale et al., 2000). Altogether, these findings point to an involvement of CRF pathways in the expression of grooming behavior. Moreover, the studies described above suggest a major role for CRF/CRFR1 circuitry in the expression and/or progressive decline (habituation) of this "displacement" behavior usually observed in rats and mice exposed to aversive environments.

Although no major alterations of anxiety-related behaviors have been detected in lifelong CRF KO mice, ICV treatment of rats with AS ON directed against CRF has been shown to attenuate the anxiogenic-like effects of social-defeat exposure as assessed in the EPM test. The behavioral results correlated with the AS-induced inhibition of the increases in hypothalamic CRF and plasma ACTH levels produced by exposure to the social-defeat experience (Skutella et al., 1994b).

Assessment of anxiety-like behaviors in null mutant mice with selective or combined inactivation of one or both of the currently known CRF receptors has further contributed to delineate the specific role each CRF receptor plays in modulating behavioral responses to stressful stimuli. Evaluation of CRFR1 KO mice in multiple behavioral paradigms has revealed a striking anxiolytic-like profile in these mice. In particular, during the LD box task, CRFR1 KO mice showed decreased latencies to enter, made more entries into, and spent more time in, the aversive brightly lit compartment as compared to WT mice (Timpl et al., 1998; Contarino et al., 1999). CRFR1 KO mice showed decreased anxiety-like responses also during performance of two other tests of anxiety, i.e., the EPM and the Defensive Withdrawal (DW) tests. The mutant mice entered more frequently and spent more time in the open arms of an EPM apparatus as well as spent more time out of the relatively "safe" dark chamber placed into an open field during the DW task as compared to WT mice (Smith et al., 1998). CRFR1 KO mice are characterized by very low, almost undetectable, plasma levels of corticosterone. Thus, the role for the glucocorticoid deficiency in the decreased anxiety-like behaviors displayed by CRFR1 KO mice was also examined in WT and CRFR1 KO mice

treated with corticosterone in the drinking water for 14 days, prior to testing in the EPM. Doses of corticosterone producing similar basal plasma levels of this glucocorticoid in WT and CRFR1 KO mice did not affect the decreased anxiety-like profile of the mutant mice, providing further support for the hypothesis that central CRF pathways modulate anxiogenic-like effects of aversive events, independent of the HPA axis (Smith et al., 1998). Finally, an important role for CRFR1 in mediating the anxiogenic-like effects of drug withdrawal has also been demonstrated. Following exposure to a forced alcohol-drinking procedure, alcohol withdrawn CRFR1-deficient mice showed higher levels of exploration of the lit compartment of an LD apparatus as compared to WT and CRFR1 heterozygote mice undergoing alcohol withdrawal (Timpl et al., 1998). These findings indicate a crucial role for CRFR1 in mediating the anxiogenic-like effects of alcohol withdrawal and suggest an important role for this CRF receptor subtype in mediating the aversive affective components of drug withdrawal.

Findings obtained with CRFR1 KO are consistent with antisense studies showing anxiolytic-like properties of CRFR1 knockdown. In particular, intra-amygdala infusion of AS ON directed against CRFR1 was shown to reduce anxiety-like behaviors assessed in the EPM test that were associated with social defeat. AS-treated rats spent more time and made more entries into the open arms of an EPM apparatus than rats treated with scrambled sequence ON. However, this study also showed very low levels of mRNA CRFR1 in the central nucleus of the amygdala of rats treated with scrambled sequence ON as well as AS, thus, making it difficult to ascribe the altered anxiety-like behaviors observed in AS-treated rats to specific CRFR1 knockdown (Liebsch et al., 1995). Another study showed anxiolytic-like effects of ICV treatment with CRFR1 AS ON in the DW test but no effect of CRFR2 AS administration. However, quantification of receptor knockdown levels revealed significant but very low decreases of frontal cortex and amygdala CRFR1. Moreover, treatment with CRFR1 AS ON did not alter the anxiogenic-like effects of a swim stressor as assessed in the EPM test and failed to inhibit the increases in plasma ACTH levels produced by the stressful swim experience (Heinrichs et al., 1997a). The combined mild CRFR1 knockdown and the toxic effects of missense and AS treatment such as body weight loss shown in the latter study might have contributed to the lack of an effect of CRF receptor knockdown in the EPM test. In contrast, others have demonstrated the ability of a chronic ICV infusion with CRFR1 AS to inhibit the anxiogenic-like effects of a social-defeat experience as assessed in the EPM test (Liebsch et al., 1999). However, in this study, treatment with AS CRFR1 did not affect plasma ACTH and corticosterone levels and specific CRFR1 knockdown was not characterized.

Interestingly, examination of CRFR2 KO mice revealed anxiety-like profiles opposite to those previously observed in CRFR1 KO mice. In particular, CRFR2-deficient mice were shown to display increased anxiety-like behaviors during both the EPM and the OF tests. However, no effect of the gene mutation was detected in the LD task (Bale et al., 2000). CRFR2 KO mice also showed increased levels of urocortin (UCN) in the Edinger-Westphal nucleus (Bale et al., 2000; Coste et al., 2000) and increased CRF levels in the central nucleus of the amygdala (Bale et al., 2000). The elevated amygdalar CRF levels observed in CRFR2 KO mice may be responsible, at least in part, for the increased anxiety-like behaviors displayed by the mutant mice. Another study demonstrated anxiogenic-like effects of the CRFR2 null mutation as assessed in the EPM test. However, whereas increased anxiety-like behaviors were observed in CRFR2 KO male mice, no difference was detected between WT and CRFR2 KO female mice (Kishimoto et al., 2000). Moreover, other authors reported no change in anxiety-like parameters in both female and male CRFR2 KO mice (Coste et al., 2000).

Behavioral profiles similar to those observed in CRFR2 null mutant mice were also obtained following treatment with a CRFR2-selective peptide antagonist, antisauvagine 30 (aSv30), or a CRFR1-preferring peptide agonist, ovine CRF (Kishimoto et al., 2000). However, with regard to the results obtained with aSv30 it should be mentioned that, more recently, treatment of rats with this peptidic CRF antagonist has been shown to produce anxiolytic-like effects in both the EPM and DW tests (Takahashi et al., 2001). It is difficult to provide an explanation for the conflicting results obtained in the rat and mouse studies mentioned above using the compound aSv30. Clearly, these discrepancies highlight the need for further studies to better characterize the behavioral profiles of the newly developed CRFR1- and CRFR2-selective antagonists. In fact, to date, results obtained with CRF receptor-selective compounds have been shown to be largely inconsistent across several studies using either the same or different animal species (Griebel et al., 1998; Jasnow et al., 1999; Okuyama et al., 1999; Habib et al., 2000; for a review see Takahashi, 2001).

Thus, findings obtained using CRFR1 or CRFR2 KO mice indicate opposite functional roles for the two currently known CRF receptor subtypes in anxiety-like behaviors. Stimulation of CRFR1 may be responsible for increased anxiety-like responses whereas activation of CRFR2 may produce anxiolytic-like effects. At the same time, results obtained in intact rats using pharmacological and antisense probes suggest more complex interactions. Relative, as well as, finely regulated contribution of the two CRF receptors may be essential in coordinating physiological responses to stressful events.

More recently, CRFR1/R2 KO mice were also examined for anxiety-like profiles. CRFR1/R2 KO mice displayed less anxiety-like behaviors than WT mice in all three different tasks in which they were tested, i.e., the EPM, OF, and LD. In particular, female CRFR1/R2 KO mice spent more time in, and entered more frequently, the open arms of an EPM apparatus than sex-matched WT mice (Bale et al., 2002). During the OF test, the double mutant female mice made more crossings into the inner squares but showed levels of ambulatory activity (total square crossings) similar to WT mice (Figure 2A and B). Finally, during the LD test, CRFR1/R2 KO female mice took less time to enter the aversive light compartment and made more transitions between the two compartments than WT mice (Figure 3A and B). However, while robust anxiolytic-like effects of the double CRFR1/R2 null mutation were observed in the female mice, examination of CRFR1/R2 KO male mice in the EPM and OF tests revealed no change in anxiety-like behaviors as compared to male WT mice (Bale et al., 2002). Moreover, this study showed that

male, but not female, mice born to heterozygote or null mutant CRFR2 dams displayed increased anxiety-like behaviors in the EPM test independent of the pup's genotype. The latter findings support a role for maternal care in "shaping" anxiety-like responses to stressors displayed by mice. However, more studies are needed to examine possible differences in maternal behaviors of CRFR2-deficient mice as compared to WT or CRFR1-deficient dams and their effects on anxiety-like profiles displayed by the pups.

The role for the CRF-BP in modulating anxiety-like responses has also been examined in pitCRF-BP TG mice. During exposure to the OF or the EPM tasks, pitCRF-BP TG mice showed higher levels of ambulatory behaviors but no difference in anxiety-like parameters as compared to WT mice. This might reflect the restricted CRF-BP over-expression to the anterior pituitary gland of pitCRF-BP TG mice without altered CRF-BP expression in the brain (Burrows et al., 1998). In contrast, altered anxiety-like behaviors were detected in CRF-BP KO mice. CRF-BP null

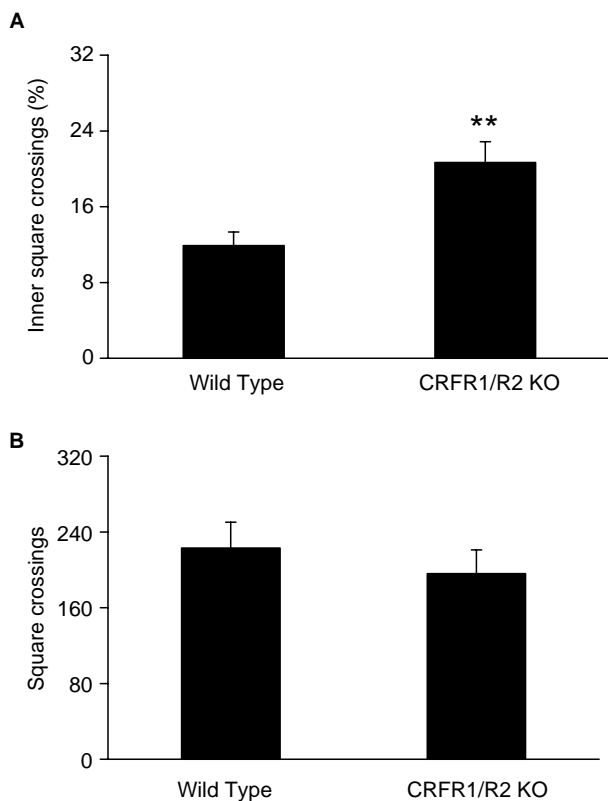


Fig. 2 Percentage of inner square crossings (A), calculated as inner square crossing/total square crossing $\times 100$, and total (inner plus outer) square crossings (B) performed by WT mice ($n = 10$) and CRFR1/R2 null mutant female mice ($n = 11$) during a 10-min Open-Field (OF) test. The OF apparatus consisted of a white Plexiglas box ($50 \times 50 \times 22$ cm, L \times W \times H) with 16 squares (12×12 cm) painted on the floor (12 outer and 4 inner). ** $P < 0.005$ versus WT mice. Student's t test. For further details see Bale et al., 2002.

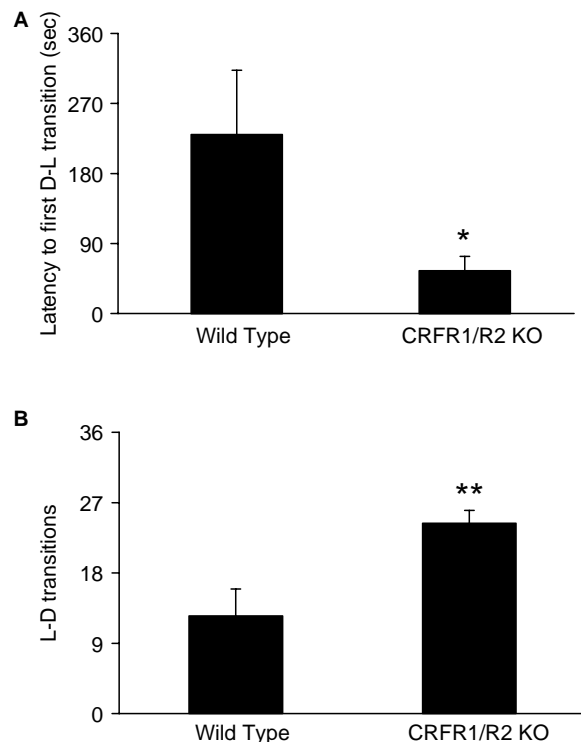


Fig. 3 Latency (sec) to first dark-light transition (A) and number of light-dark transitions (B) performed by WT mice ($n = 10$) and CRFR1/R2 null mutant female mice ($n = 11$) during a 10-min Light-Dark (LD) box test. The LD apparatus was a rectangular Plexiglas box divided by a partition into one black compartment ($14.5 \times 27 \times 26.5$ cm, L \times W \times H) covered by a red Plexiglas lid (8 lux) and a white compartment ($28.5 \times 27 \times 26.5$ cm, L \times W \times H) highly illuminated (400 lux). An opening (7.5×7.5 cm) in the partition allowed transitions between the two compartments. * $P < 0.05$, ** $P < 0.005$ versus WT mice. Student's t test. For further details see Bale et al., 2002 and Contarino et al., 1999.

mutant mice spent less time and made fewer entries into the open arms of an EPM apparatus than WT mice. Moreover, during the DW test CRF-BP KO mice exited the protected darkened chamber of a brightly lit arena less frequently than WT mice (Karolyi et al., 1999). These findings indicate anxiogenic-like effects of circulating CRF liberated due to CRF-BP-deficiency.

COGNITION

Multiple lines of evidence indicate a role for the CRF system in the neural pathways underlying learning and memory processes. In Alzheimer's disease (AD) patients, CSF levels of CRF may correlate with the degree of cognitive impairment in that greater cognitive deficits are associated with lower CSF CRF concentrations (Pomara et al., 1989). Cerebral cortex tissues derived from AD and Parkinson's disease patients contain reduced CRF concentrations that correlate with decrements in choline acetyltransferase (ChAT) activity, the key enzyme for acetylcholine synthesis (De Souza et al., 1986; Whitehouse et al., 1987). Thus, altered CRF levels might contribute to memory impairments observed in neurological disorders. Memory deficits and reduced hippocampal volume also correlate with elevated plasma levels of cortisol in human aging (Lupien et al., 1998). In addition, animal studies provide further support in favor of a role for CRF pathways in cognitive processes. Treatment with CRF can improve learning performance in rats (Koob and Bloom, 1985) and increases in cognitive abilities have also been observed in rats treated with human CRF₆₋₃₃, a CRF-BP ligand that increases the "free" active concentrations of CRF (Behan et al., 1995).

Mouse models with a selective gene mutation provide the unique possibility to gain further insight into the role for a specific element of the CRF system in cognitive processes. Thus, in line with the notion of an inverted U-shaped relationship between brain CRF levels and memory performance (Koob, 1991), CRF TG mice were shown to display impaired learning and memory abilities. In particular, during the Morris water-maze test CRF TG mice were slower than WT control mice in solving the maze and did not display any improvement over subsequent experimental days. Moreover, treatment with the benzodiazepine, chlordiazepoxide, during the acquisition phase of the task significantly improved memory retention in CRF TG mice tested one week later (Heinrichs et al., 1996). This points to a causal role for the hyper-emotional phenotype of CRF TG mice in their poor cognitive performance.

Whereas brain CRF overabundance results in cognitive impairments, CRF-deficiency has been shown to produce no alterations in learning and memory abilities. During a fear conditioning experiment, CRF KO mice displayed

levels of cognitive performance similar to those observed in WT mice (Weninger et al., 1999). However, it should be mentioned that, in this study, mice underwent an extensive training protocol (36 foot-shocks) that may have rendered it difficult to detect genotype differences. In contrast, intra-hippocampal administration of AS ON directed against CRF was shown to impair memory retention performance of rats in a passive avoidance paradigm. Moreover, behavioral effects of the AS treatment were correlated with the decreased levels of mRNA CRF observed in the hippocampus of CRF AS-treated rats but not in sense-treated control rats. Intra-hypothalamic administration of CRF AS also produced significant decreases in plasma ACTH and hypothalamic mRNA CRF levels that were not present in sense-treated controls (Wu et al., 1997).

Further support for an important role for CRF pathways in cognitive functions was provided by the findings of disrupted memory processes in CRFR1 KO mice. During the retrieval trial of a Y-maze test, CRFR1 null mutant mice showed no increase in exploration of the novel arm (Contarino et al., 1999), indicating impaired spatial recognition processes as assessed in this behavioral paradigm (Dellu et al., 1992; Conrad et al., 1996). Performance in the Y-maze task has been shown to depend on the hippocampal formation (Conrad et al., 1996), a brain region with a large distribution of CRFR1 (Chalmers et al., 1995). Thus, lack of CRFR1 in the hippocampus and other brain regions subserving spatial memory processes may be responsible, at least in part, for the poor memory performance displayed by CRFR1 KO mice.

Impairments have also been observed following treatment of rats with the non-peptide CRFR1-selective antagonist antalarmin. In particular, administration of this CRF antagonist either before conditioning trials or testing sessions inhibited rat freezing behaviors as assessed in a fear conditioning paradigm (Deak et al., 1999). More recently, similar findings have been obtained using the CRFR2-selective antagonist aSvq-30. During a fear conditioning experiment, rats treated with aSvq-30 displayed lower levels of contextual fear responses than vehicle-treated rats (Takahashi et al., 2001). Accordingly, ICV treatment with AS ON to CRFR2 decreased brain mRNA CRFR2 levels and reduced the amount of time engaged in freezing behavior by rats exposed to contextual cues previously paired with the aversive foot-shock experience (Ho et al., 2001). The latter study also demonstrated that combined inhibition of CRFR2 and CRFR1 produced levels of conditioned freezing in rats lower than those observed following treatment with either CRFR2 AS or CRFR1-selective antagonist alone (Ho et al., 2001). Finally, studies examining region-specific effects of CRF showed that intra-hippocampal or intra-septal administration of human/rat CRF (h/rCRF) increases or decreases,

respectively, learning performance of mice tested in the fear conditioning paradigm. Moreover, using astressin and aSv3-30 it was shown that h/rCRF increased learning through CRFR1 and produced the opposite effect through CRFR2 (Radulovic et al., 1999). Decreased freezing measured in the conditioned fear paradigm may reflect either a reduction in the saliency of the shock unconditioned stimulus or a deficit in learning the association between the unconditioned stimulus and the conditioning environment or a combination of these elements. Thus, impairments measured in tests of fear-motivated behaviors must be viewed in light of the involvement of the CRF system in both the anxiety and learning components that underlie fear conditioning.

FEEDING

Several studies indicate a major role for CRF pathways in the regulation of ingestive behaviors (for a review see Heinrichs and Richard, 1999). ICV injection of CRF-like peptides produces strong anorectic effects in rats and mice (Krahn et al., 1988; Spina et al., 1996; Heinrichs et al., 2001). Moreover, CRF circuitry mediates stress-induced inhibition of consumatory behaviors (Krahn et al., 1986). Human studies also suggest an important role for CRF pathways in appetite regulation. Increased levels of CSF CRF have been detected in underweight anorexics and a normalization of CSF CRF levels has been observed following reversal of body weight loss (Kaye et al., 1987; Kaye, 1996).

Knockout and transgenic mice with altered levels of CRF or ligand sites for CRF (receptors, CRF-BP, etc.) allow the investigation of the specific role for each element of the CRF system in modulating ingestive behaviors. PerCRF-BP TG mice with widespread expression of the transgene in the brain and peripheral tissues displayed alterations in the profile of weight gain. In particular, 1-year old female perCRF-BP TG mice showed body weight values significantly higher than those detected in sex-matched WT mice. Genotype differences in body weight gain occurred between 8 and 12 months, since 8-month old transgenic mice did not differ from WT mice. A tendency to increased body weight gain was also observed in male perCRF-BP TG mice (Lovejoy et al., 1998). Alterations in the amount of food ingested may have accounted for the genotype-dependent differences in body weight gain. However, in the latter study food intake was not measured. Amount of food ingested as well as patterns of feeding behaviors were monitored in pitCRF-BP TG mice. Although no genotype differences were detected in 24-h food intake and body weight, examination of circadian patterns revealed that pitCRF-BP TG mice consumed more food during the light period and less food during the dark period of the light–dark cycle than

WT mice (Burrows et al., 1998). Finally, consistent with the anorectic effects of exogenously administered CRF, decreased weight gain and a trend toward reduced daily food intake were observed in male, but not female, CRF-BP KO mice as compared to WT mice (Karolyi et al., 1999).

Patterns of circadian food intake similar to those detected in pitCRF-BP TG mice have also been detected in CRFR1 KO mice. No genotype difference was observed in 24-h food intake; however, CRFR1 KO mice were shown to ingest more of a water-based liquid diet during the light portion of the light–dark cycle and to consume less during the dark phase as compared to WT mice. Moreover, rescue of normal basal levels of plasma corticosterone in CRFR1 KO mice resulted in normalized circadian patterns of food intake (Muller et al., 2000a). Hypothalamic CRF overexpression detected in both CRFR1 KO and pitCRF-BP TG mice might underlie the altered circadian rhythms of food intake observed in these mutant mice. Increased negative feedback stimuli by circulating glucocorticoids might, thus, reduce hypothalamic CRF levels and reinstate normal circadian patterns of feeding behaviors, such as those observed in corticosterone-treated CRFR1 KO mice. Thus, in addition to the largely reported role for brain CRF in basal and stress-induced modulation of feeding behavior, the studies mentioned above provide initial evidence in favor of a role for CRF pathways in the regulation of circadian rhythms of consumatory behaviors.

CRFR1 KO mice have also been very useful to examine the specific involvement of CRFR1 or CRFR2 in CRF- and UCN-induced anorexia. ICV treatment with CRF equally suppressed food–water intake of WT and CRFR1 KO mice during a 12-h time period following peptide dosing (Contarino et al., 2000). Similar findings were obtained with UCN (Contarino et al., unpublished results). However, despite the effect of CRF to inhibit consumatory behaviors of CRFR1 KO mice, the same peptide dose increased ambulatory levels of WT mice but did not produce any motor activation in CRFR1-deficient mice (Contarino et al., 2000). These findings indicate a crucial role for CRFR1 in the neural pathways underlying CRF-mediated arousal states and suggest the involvement of CRF receptors other than CRFR1 in mediating food intake suppression induced by CRF-like peptides.

Urocortin effects on food and fluid intake by WT and CRFR1 KO mice have also been examined by Bradbury and colleagues (2000). In agreement with the study described above, WT and CRFR1 KO mice did not differ in body weight, food or water intake during *ad libitum* conditions. Moreover, treatment with corticosterone in the drinking water did not induce any change in these parameters. Exposure to a 7-day continuous infusion of UCN suppressed food and water intake in WT and CRFR1 KO mice similarly only during the second day of peptide

treatment, indicating development of tolerance to the anorectic effects of UCN. Finally, acute injection of UCN suppressed 24-h food intake in both WT and CRFR1 KO mice. However, during the initial 1.5 h following peptide dosing, UCN significantly inhibited food consumption in WT mice without affecting food intake of CRFR1 KO mice. Starting from 3 hours after UCN administration, WT and CRFR1-deficient mice displayed comparable levels of UCN-induced anorexia (Bradbury et al., 2000). It should be mentioned that, in this study, mice had been food deprived for 17 hours before UCN dosing. In this context, it might be argued that during the initial 1.5-h following UCN treatment CRFR1 KO mice were less sensitive to the anorectic effects of the combined food deprivation and peptide dosing than WT mice. Several reports have, in fact, demonstrated a major role for brain CRF pathways in mediating stress effects on food intake behaviors (Krahn et al., 1986; Shibasaki et al., 1988). Moreover, treatment with a CRFR1-selective antagonist has been shown to reverse emotional stress-induced inhibition of food intake in rats (Hotta et al., 1999).

To date, examination of CRF null mutant mice has revealed no effect of this gene mutation on both basal levels of food intake and stress-induced inhibition of feeding behaviors. In particular, CRF KO mice did not differ from WT mice in the amount of milk ingested both under basal conditions or following exposure to a 30-min restraint stress. Moreover, treatment with the anorectic compounds lipopolysaccharide, mouse interleukin-1 β , and the serotonin releaser d-fenfluramine produced similar levels of milk- or food-intake suppression in both WT and CRF KO mice (Swiergiel and Dunn, 1999). Finally, WT and CRF KO mice displayed similar levels of adrenalectomy-induced anorexia. Rescue of corticosterone plasma levels above the circadian peak completely abolished adrenalectomy effects in both genotypes, suggesting an important role for glucocorticoids in the modulation of ingestive behaviors independent of CRF (Jacobson, 1999).

More recently, the specific role for CRFR2 in the anorectic effects of UCN was examined in CRFR2 KO mice. During the initial 4-h after UCN dosing, comparable levels of food-intake inhibition were observed in WT and CRFR2-deficient mice. However, while UCN strongly suppressed food intake in WT mice during the entire 10-h test, starting from six hours after the peptide treatment, no anorectic effects were evident in UCN-treated CRFR2 KO mice (Coste et al., 2000). These findings suggest that whereas CRFR2 may not be involved in the early hypophagic effects of UCN, this CRF receptor subtype plays a crucial role in mediating the late-phase suppression of food intake by the peptide. Moreover, since mice were food deprived for 16 h prior to UCN treatment, together with prior findings (Bradbury et al., 2000) these results

suggest that, unlike CRFR2, CRFR1 is the main CRF receptor mediating the anorectic effects of stress exposure.

Thus, the studies described above strongly support an important role for CRFR2 in the anorectic effects of CRF-like peptides. Involvement of CRFR2 in modulating feeding behaviors is further highlighted by studies employing antisense techniques and the CRFR2-selective antagonist aSv30. In particular, treatment of rats with AS ON against CRFR2 inhibited both CRF- and UCN-induced suppression of food intake whereas aSv30 was shown to antagonize CRF anorectic effects in mice (Smagin et al., 1998; Pelleymounter et al., 2000). Finally, in contrast with previous rat studies (Spina et al., 1996), more recently, it has been shown that CRF produces higher levels of food intake suppression than UCN in mice. In addition, treatment with CRF, but not UCN, increased brown adipose fat and adrenal weights as well as reduced thymus and spleen size. Interestingly, aSv30 effectively inhibited CRF anorectic properties but did not modify its metabolic effects (Cullen et al., 2001).

SUMMARY

The creation of mice with genetic modifications resulting either in overexpression or deletion of critical mediators of CRF neuropeptide function represents additional experimental models for the investigation of physiological and behavioral regulation. Targeted manipulations of specific CRF system elements (peptide, receptors, binding protein, etc.) have proven very useful to understand HPA axis biological actions during both normal and pathological conditions (Table 1). Specifically, data from receptor null mutant mice suggest a crucial role for CRFR1 in mediating HPA endocrine responses to stressful stimuli and indicate that stimulation of CRFR2 might counterbalance CRFR1-mediated effects. Results obtained using CRFR1 or CRFR2 KO mice indicate opposite functional roles for the two currently known CRF receptor subtypes in anxiety-like behaviors as well. Relative as well as finely regulated contribution of the two CRF receptors may be essential in coordinating physiological responses to stressful events. Mouse models with a selective gene mutation have also provided further insight into the role for specific elements of the CRF system in cognitive processes. The importance of CRF pathways in cognitive functions was demonstrated by the findings of disrupted memory processes in CRFR1 KO mice and cognitive impairments, albeit related to hyper-emotionality, in CRF TG mice. Moreover, knockout and transgenic mice with altered levels of CRF or ligand sites for CRF (receptors, CRF-BP, etc.) have facilitated the investigation of the specific contribution of each element in modulating ingestive behaviors. Beyond an involvement for brain CRF in basal and stress-induced modulation of feeding behavior, the studies reviewed provide

Table 1 CRF System Mutant Mice.

Over-Expression	Physiological Phenotype	Behavioral Phenotype	Reference Citation ^a
CRF TG Metallothionein	Elevated plasma ACTH & corticosterone Hypertrophy of adrenal gland Attenuated HPA response to stress Cushingoid features	Increased anxiety-like behavior Impaired learning Diminished female sexual receptivity	Stenzel-Poore et al., 1992
CRF TG Thy-1	Elevated plasma corticosterone Hypertrophy of adrenal gland Dexamethasone non-suppression	Reduced startle reactivity with impaired habituation Impaired pre-pulse inhibition ^b	Groenink et al., 2002
pitCRF-BP TG perCRF-BP TG	Increased hypothalamic CRF & VP Normal levels of ACTH & corticosterone	Circadian alterations in food intake Late onset weight gain in females	Burrows et al., 1998 Lovejoy et al., 1998
Deletion			
CRF KO	Adrenocortical atrophy Lack diurnal rise in plasma corticosterone Blunted HPA response to stress	No alterations in basal or stressed anxiety behaviors Increased grooming	Muglia et al., 1995
CRF-BP KO	Normal HPA response to stressful stimuli	Increased anxiety-like behavior Decreased weight gain in males	Karolyi et al., 1999
CRFR1 KO	Decreased plasma corticosterone Atrophy of adrenal subregions Upregulation of ACTH secretagogues Eliminated HPA response to stressful stimuli	Increased grooming Reduced anxiety-like behavior Impaired spatial recognition memory Altered circadian pattern of food intake Insensitive to motor activation by CRF	Smith et al., 1998; Timpl et al., 1998
CRFR2 KO	Normal levels of ACTH & corticosterone Enhanced HPA response to stressful stimuli	Increased anxiety-like behavior Normal grooming Disrupted late phase suppression of feeding by UCN	Bale et al., 2000; Coste et al., 2000
CRFR1/R2 KO	Decreased plasma corticosterone Atrophy of adrenal glands Eliminated HPA response to stressful stimuli	Increased grooming Decreased anxiety-like behaviors in females	Preil et al., 2001; Bale et al., 2002

^aReference citation for creation of mouse; ^bDirks et al., 2002.

initial evidence in favor of a role for CRF pathways in the regulation of circadian rhythms of consumatory behaviors. In summary, targeted mutations of the CRF system represent an important tool for the further investigation of regulation of physiology and behavior by this neuropeptide.

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