Neuropeptide Regulation of Stress-Induced Behavior: Insights from the CRF/Urocortin Family

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INTRODUCTION: THE CRF FAMILY OF PEPTIDES AND RECEPTORS

Perception of physical or psychological stress by an organism is followed by a series of events that result in changes in emotional and cognitive functions, modulation of autonomic activities and the secretion of corticosteroids from the adrenal cortex. The intracerebral administration of CRF or Ucn1 results in behavioral responses that are similar to those observed when animals are exposed to a stressor. The behavioral effects include increased anxiety-like behavior, decreased food consumption, increased arousal, altered locomotor activity, diminished sexual behavior, and sleep disruption. The role of the CRF/Urocortin system in modulating the behavioral responses to stressors was further demonstrated based on the behavioral phenotypes of transgenic mice that overexpress, or are deficient in, the different CRF family members. The CRF–CRFR1 system is suggested as critical for initiating stress responses, while the urocortins–CRFR2 system is suggested to terminate stress responses and restore homeostasis.

INTRODUCTION: THE CRF FAMILY OF PEPTIDES AND RECEPTORS

Perception of physical or psychological stress by an organism is followed by a series of events that result in changes in emotional and cognitive functions, modulation of autonomic activities and the secretion of glucocorticoids from the adrenal cortex. Both the activation
and the termination of the behavioral, autonomic and adrenocortical stress responses are crucial for adaptation and survival. The neuropeptide corticotropin-releasing factor (CRF), expressed and secreted from the paraventricular nucleus (PVN) of the hypothalamus, represents the final common pathway for the integration of the neuroendocrine stress response in the brain. CRF and, to a lesser extent, arginine vasopressin (AVP) play an important and well-established role in the regulation of the hypothalamus–pituitary–adrenal (HPA) axis under basal and stress conditions. In addition to the PVN, CRF mRNA and peptide have been found in several extrahypothalamic brain nuclei, including the cerebral cortex, amygdala, bed nucleus of stria terminalis (BNST), and hippocampus, suggesting that, in addition to its hypophysiotropic action, CRF acts to integrate the neuroendocrine, behavioral, autonomic and metabolic responses to stressors. Dysregulation of the stress response can have severe psychological and physiological consequences, and chronic hyperactivation of the CRF system has been linked to stress-related emotional disorders such as anxiety, anorexia nervosa and melancholic depression. In addition to CRF, the mammalian CRF-peptide family contains urocortin (Ucn) 1, 11 Ucn2, 12, 13 and Ucn3, 13, 14 CRF and its related peptides signal through the activation of two high-affinity G-protein coupled receptors, CRF receptor type 1 (CRFR1) 15–17 and CRFR2. 18–22 Both CRFR1 and CRFR2 belong to the class B1 subfamily of seven-transmembrane domain receptors that signal by coupling to G proteins. CRFR1 has one known functional splice variant (α) expressed in the central nervous system and the periphery. The CRFR1 mRNA is widely expressed in mammalian brain and pituitary, with high levels in the anterior pituitary, cerebral cortex, cerebellum, globus pallidus, amygdala, hippocampus and olfactory bulb. In the periphery, CRFR1 is expressed in testes, ovary, skin and spleen. CRFR2 has three functional membrane splice variants in the human (α, β and γ) and two in the rodent (α and β). 18–22, 24, 26 CRFR type 2α (CRFR2α) is the major splice variant in the rodent brain. It is expressed in a more discrete pattern than CRFR1, with highest densities in the lateral septum, BNST, ventromedial hypothalamic nucleus (VMH), olfactory bulb and mesencephalic dorsal raphe nucleus (DRN) whereas CRFR type 2β (CRFR2β) is expressed primarily in peripheral tissues and the choroid plexus of the brain. CRFR1 and CRFR2 differ pharmacologically: CRF has relatively lower affinity for CRFR2 compared to its affinity for CRFR1, Ucn1 has equal affinities for both receptors, and Ucn2 and Ucn3 appear to be selective for CRFR2. 11, 12, 14 In addition to both CRF receptors, CRF and Ucn1 can bind to the CRF-binding protein (CRF-BP) suggested to function as an endogenous “buffer,” thus adding a further level of complexity to the control of the CRF-related ligands’ actions. 28–30

III. HORMONES, BRAIN FUNCTION AND BEHAVIOR

CENTRAL ADMINISTRATION OF CRF/Ucn1

The intracerebroventricular administration of CRF or Ucn1 results in behavioral responses that are similar to those observed when animals are exposed to a stressor (Fig. 15.1). The behavioral effects include increased anxiety-like behavior, decreased food consumption, increased arousal, altered locomotor activity, diminished sexual behavior, and sleep disruption (for detailed reviews, see references 5, 31–35).

One of the earliest and best described effects of central CRF administration is increased arousal and altered locomotor activity which is dependent on the testing conditions. In non-stressed rats tested in a familiar environment, i.c.v. injection of CRF elicits a dose-dependent increase in general behavioral activation that includes elevated walking, excessive grooming and rearing. This effect is apparently mediated by central pathways and independent of pituitary–adrenal activation, since CRF increases locomotor activity in both naïve and hypophysectomized animals, and the effect is not blocked by dexamethasone administration. The potent stimulatory effect of CRF in behavioral activation is evolutionary conserved, as has also been described in several vertebrate species. CRF increases swimming and walking in amphibians, and fish, stepping in birds, and general motor activity in pigs, mice and primates.

In contrast to the behavioral activating effect of CRF in animals under low arousal conditions, when animals are exposed to a novel stressful environment such as an open field or an elevated plus maze, CRF administration effects changes to behavioral inhibition. Following i.c.v. injection of CRF, rodents show decreased locomotion, rearing and inner square crossings in an open field test, a reduced percentage of time spent on the open arms of the elevated plus maze, and decreased investigatory behavior in a multi-compartment chamber test. In addition to the behavioral suppression observed in these exploration tests, the anxiogenic-like profile of central CRF administration includes increased stress-induced freezing behavior, reduced social interaction, potentiated acoustic startle response, and increased defensive burying. Other stress-related behaviors induced by i.c.v. CRF administration are decreased food consumption, inhibition of maternal behavior, decreased sexual behavior, and the induction of place and taste aversion.
BOX 15.1

HOW DO WE KNOW THE GENETIC APPROACH FOR INTRACEREBROVENTRICULAR DELIVERY?

Pharmacological administration of synthetic peptides or secreted recombinant proteins into the brain ventricles is a common method used by neuroscientists for exploring physiological and behavioral functions of novel or known gene products. Cerebroventricular (rather than systemic), administration of these proteins is required in order to bypass the blood–brain barrier (BBB) and allow the non-selective transport of peptides or proteins from the periphery into the central nervous system (CNS).

Current approaches for delivery of peptides or secreted proteins to the CNS include, for short-term acute administration, a stereotaxic injection into the ventricular space, known as intracerebroventricular (i.c.v. administration); and for chronic delivery, an i.c.v. microinjection pump. These methods rely heavily on the solubility and half-life of the injected substance, require chemical or in vitro synthesis of the administered ligand, and may involve different purification procedures. The i.c.v. administration is difficult to use in studies requiring repeated or prolonged administration of the ligand, since the microinjection pump procedure depends on the ligand stability and capacity of the reservoir, and complex surgical procedures are required for installation and manipulation of the pump. Furthermore, the current procedures require (Continued)
extensive handling of the experimental animals, which may create behavioral or physiological disturbances.

We have recently described a genetic approach for the delivery of secreted peptides or proteins into the cerebrospinal fluid (CSF) using a choroid plexus-specific and lentiviral-based genetic system. The choroid plexus plays a critical role in the barrier mechanism regulating the exchange of molecules between the brain’s internal milieu and the periphery. This blood–CSF barrier is composed of epithelial cells with apical tight junctions that restrict intercellular passage of molecules from fenestrated blood vessels. The CSF circulatory system’s function is to provide micronutrients, neurotrophins, hormones, neuropeptides and growth factors extensively to neuronal networks. Therefore, neuromodulators directed to CSF can modify and adapt a variety of behavioral, neuroendocrine and immunologic processes.

Using a choroid plexus-specific promoter, we established a lentiviral-based system, which offers inducible and reversible delivery of a gene product into the CSF. The system is composed of two complementary lentiviral vectors. The “Effector” construct consists of a choroid plexus-specific promoter that drives the expression of reverse tetracycline transactivator (rtTA) protein and the reporter green fluorescent protein (GFP) (see A, upper panel). The “Target” construct includes the tetracycline-responsive element (TRE) DNA sequence, upstream to the nucleotide coding sequence of the requested gene of interest, followed by the reporter red fluorescent protein (RFP) (see A, lower panel). Transcription initiation of the gene of interest

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**BOX 15.1 (cont’d)**

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and the RFP is mediated only in the presence of the inducer, doxycycline (Dox). A mixture of the two lentiviruses is injected intracerebroventricularly, and the delivered genes are incorporated into the DNA of the choroid plexus cells. Initiation of transcription, limited to the choroid plexus cells by the choroid plexus-specific promoter, is induced by administrating Dox-containing drinking water, and results in secretion of the final processed gene product into the CSF (see B). Dox is the inducer of choice for our purposes, as it has been demonstrated to cross the blood–brain barrier. The functionality of this system was demonstrated using the overexpression of the two established neuropeptides, corticotropin-releasing factor and gonadotropin-releasing hormone, modulating anxiety-like behavior and estrous cycle, respectively.1

Reference

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administration of Ucn1 results in a similar behavioral profile to that of CRF, including increased grooming and motor activity in a familiar environment,38 and increased anxiety-like behavior in different paradigms, including the open field test, the elevated plus maze test, the light–dark transfer test, the defensive withdrawal test and the conflict test.76,77

Recently, a new genetic approach has been applied for the inducible and reversible secretion of CRF into the cerebrospinal fluid (CSF).78 The choroid plexus, a group of modified ependymal cells in the ventricles of the brain that produce the CSF, was genetically targeted by i.c.v. injection of a lentiviral vector expressing CRF. The initiation of CRF transcription, limited to the choroid plexus cells by a choroid plexus-specific promoter, was induced by administrating the animals with doxycycline in the drinking water, which results in the secretion of the CRF peptide into the CSF. Mice conditionally overexpressing CRF at the choroid plexus showed an increase in anxiety-like behavior in the light–dark transfer, open field and elevated plus maze tests relative to the control group.78 These results further support the principal role of CRF in the regulation of anxiety, previously demonstrated by pharmacological studies.
CNS SITES OF ACTION OF CRF/UCN1

Amygdala

The amygdala is an important mediator of fear and anxiety. In view of the relative high concentration of CRF in the central nucleus and the dense concentration of CRF receptors in the basal nucleus, the amygdala has been largely investigated for its role in the effects of CRF on emotion-related behavior. In rats, CRF injected into the central amygdala (CeA) induces an increase in locomotor activity in a familiar environment, but a decrease in locomotion in a novel open field — results that resemble those obtained by i.c.v. administration of CRF. CRF or Ucn1 injected into the basolateral amygdala (BLA) reduced social interaction time, led to a persistent increase in anxiety-like behavior, decreased feeding, and increased grooming, suggesting a specific role for the amygdalar CRF system in anxiety-like behavior. Two research groups have recently used a lentiviral-based system for chronic amygdala-specific overexpression of CRF. Keen-Rhinehart and colleagues found that female rats overexpressing CRF in the amygdala for a period of 2 weeks showed an increase in acoustic startle response and attenuated sexual motivation, both results indicating an increase in anxiety levels. Regev and colleagues found that male mice overexpressing CRF in the amygdala for a period of 4 months showed reduced levels of anxiety-like behavior in response to acute stressful stimulation in the open field and light–dark transfer tests.

The discrepancy between the studies may be attributed to the difference in CRF overexpression duration, gender differences, species differences and behavioral tests. Injections of α-hCRF9–41, a synthetic competitive antagonist of CRF receptor, into the CeA reverses the social stress-induced decrease in time spent in the open arms of the EPM and attenuates stress-induced freezing behavior. Intra-CeA infusions of antisense oligodeoxynucleotides against CRFR1 reduce anxiety-like behavior in socially defeated rats. Intra-BLA administration of astressin B, a CRFR1/2 antagonist, reverses the anxiogenic effects of Ucn1, whereas intra-BLA administration of the CRFR1-specific antagonist antalarmin prevents defeat-induced defensive behavior in mice. Finally, a recent study has demonstrated that the decrease in anxiety-like behavior in mice reared in an enriched environment is associated with very low levels of CRFR1 in the BLA. Knockdown of CRFR1 in the BLA mimicked the anxiolytic effect of environmental enrichment.

BNST

Heavily innervated by the amygdala, and projecting to several regions involved in fear and anxiety responses, the BNST is considered an additional brain site that potentially mediates the behavioral effects of CRF/Ucn administration. Expressing CRF and both CRF1 and CRF2 receptors, the BNST has been suggested to play a special role in longer-duration anxiety-like responses, in contrast to the CeA implicated in shorter-duration fear responses. Microinfusions of CRF into the BNST increase startle amplitude in a dose-dependent manner. In the same study, intra-BNST injection of α-hCRF9–41 attenuated the effect of CRF injected i.c.v. in the startle response test. Moreover, “priming” with subanxiogenic doses of Ucn1 in the BNST elicits a persistent anxiogenic profile in the social interaction test and the elevated plus maze test, an effect blocked by prior local injection of CRFR1 antagonists. In another study, post-training administration of CRF in the BNST enhanced retention in an inhibitory avoidance task, suggesting a role for the CRF system in the BNST in memory formation processes for affective experience. The BNST is also involved in CRF-induced anorexia, as CRF administrated directly into the BNST, but not the CeA or the LC, induces a marked inhibition of feeding.

PVN

The paraventricular nucleus of the hypothalamus (PVN) contains a large number of CRF-immunopositive cell bodies and fibers, as well as moderate concentrations of CRF receptors. In addition to its role in activating the HPA axis by its projections to the median eminence, the CRF neurons in the PVN may be involved in CRF-induced behavioral inhibition through its projections to brainstem nuclei. CRF administration directly into the PVN induces a dose-dependent increase in grooming, and an increase in locomotion at lower doses but a suppression of locomotion at higher doses of CRF. In addition, intra-PVN injections of CRF decrease food intake, whereas α-hCRF9–41 injections in the PVN potentiate feeding induced by NPY injected in the same locus. A recent study has demonstrated that lentiviral-mediated knockdown of CRF in the PVN attenuates social avoidance in chronic social-defeated mice.

Hippocampus

The hippocampus contains scattered CRF-stained interneurons and moderate concentrations of CRF receptors. The dorsal hippocampus has been suggested to be involved in context-dependent and tone-dependent fear conditioning. Injections of CRF in the
dorsal hippocampus before or after training enhance both context-dependent and tone-dependent fear acquisition. This effect seems to be CRFR1-mediated, as it is prevented by local injections of astressin B, a non-selective CRF receptor antagonist, but not by antisauvagine-30, a specific CRFR2 antagonist. Furthermore, recovery from stress-induced increased anxiety and impairment of context-dependent fear conditioning requires hippocampal CRFR1, as the recovery is prevented by intra-hippocampal injections of DMP696, a highly selective CRFR1 antagonist, but not by antisauvagine-30. Finally, the ventral hippocampus has been implicated in the modulation of anxiety-related behaviors. Micro-infusions of CRF into the ventral hippocampus increase anxiety-like behavior in the EPM test, as well as stress-induced freezing.

**Locus Coeruleus**

Both physiologic and psychogenic stressors activate the noradrenergic cells in the locus coeruleus (LC). These cells project to the forebrain and the spinal cord, and induce a state of arousal required for the correct behavioral and physiological response to stress. Several studies suggest that CRF plays an important role in this effect. CRF infused into the LC increases non-ambulatory motor activity (shifting in body position), whereas locomotor activity is not altered. In addition, CRF infused into the LC produces a dose-dependent decrease in floating behavior in a modified Porsolt swim test, whereas struggling behavior is not affected. Both results suggest an increased arousal and agitation effect of CRF in the LC. In the same study, CRF was injected into the LC increased inner and outer crossings, free and wall rearing, as well as exploration outside of a darkened compartment in an open field test, indicating an anxiogenic effect. Furthermore, injection of α-hCRF9–41 into the LC reduces the levels of shock-induced freezing behavior and immobilization stress-induced defensive withdrawal. Finally, CRF injected directly into the LC improves memory retention in a passive-avoidance task, suggesting involvement of the LC in the learning and memory-facilitating effects of CRF.

**THE CRFR2/UROCORTIN CENTRAL SYSTEM**

The CRF-peptide family includes, in addition to CRF, the three urocortin (Ucn) peptides (Ucn1, Ucn2 and Ucn3) that bind and activate the CRF receptor type 2 (CRFR2) with high affinity. CRF has a relatively lower affinity for CRFR2 than for CRFR1, Ucn1 has equal affinities for both, and Ucns 2 and 3 appear to be selective for CRFR2. These receptors are distributed differently throughout the brain, while CRFR1 is widely expressed, CRFR2 expression is more localized to selected stress-related brain nuclei, such as the medial amygdala, the bed nucleus of the stria terminalis (BNST), the lateral septum (LS) and the dorsal raphe nucleus (DRN).

While the role of CRF and CRFR1 in the activation of the HPA axis and the regulation of emotional and cognitive functions following exposure to stressful challenge are well established, the role of CRFR2 is still controversial. Studies exploring the role of CRFR2 using knockout mice models, antisense oligonucleotide, or antagonist administration are less clear, and show conflicting behavioral results. Central administration of Ucn2 or Ucn3, CRFR2 antisense oligonucleotide, and CRFR2 agonists have shown contrasting, dose-dependent and localization-dependent results. These results suggest a central role for CRFR2 that may vary between different brain nuclei and under different stress conditions. Several pharmacological studies suggest anxiolytic-like effects of CRFR2 activation. Intracerebroventricular administration of Ucn2 or Ucn3 or CRFR2 agonists in the septum was shown to vary under different stress conditions, being less involved under low stress conditions, and significantly increasing anxiety-like behavior under high stress conditions.

A growing body of evidence suggests that Ucn1 and Ucn2 may influence stress-related physiology and behavior by modulating the DRN serotonergic system. Ucn2 i.c.v. administration caused an increase in c-Fos immunostaining in topographically organized subpopulations of serotonergic neurons in the DRN, specifically within the dorsal part of the mid-rostrocaudal DRN (DRD) and the caudal part of the DRN (DRC). Moreover, while injection of Ucn2 to the DRC leads to increased 5-HT release in the BLA and potentiation of conditioned fear, as well as escape deficits in a model of learned helplessness, injection of the CRFR2 antagonist antisauvagine-30, and not of CRFR1 antagonists, showed anxiolytic effects, including reversal of the potentiation of conditioned fear and the escape deficits following exposure to inescapable stress. Thus, these behaviors indicating heightened anxiety in response to uncontrollable stress seem to be mediated by CRFR2 receptors on serotonergic neurons in the DRC.
GENETICALLY ALTERED MICE

A major approach for the study of the involvement of the CRF/urocortin system in the regulation of stress-induced behavior has been the use of transgenic mice models, overexpressing or knocking out the different family members. To date, all genes of the CRF/urocortin family have been successfully targeted, generating deficient animal models that have been crucial for the understanding of the role of the CRF/Urocortin family of peptides and receptors in mediiating stress-related behaviors.

In the case of developmental transgenic mice model, it should be remarked that conclusions regarding their physiology and behavior must be drawn cautiously, due to compensatory changes in other related or non-related genes. In addition, in the case of gene overexpression the expression of the gene of interest is often driven under the control of a general promoter, resulting in overexpression of the gene of interest in non-endogenous brain regions and peripheral organs (for detailed reviews on CRF family mutant mice, see references 161–163) (Table 15.1).

CRF-OE

To study the effects of chronic exposure to CRF, transgenic mice overexpressing CRF under the control of

<table>
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<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference(s)</th>
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<tr>
<td>CRF OE (Stenzel-Poore et al.)</td>
<td>High plasma levels of ACTH and corticosterone. Increased anxiety and depression-like behavior, decrease in sexual behavior (only in females) and deficits in learning and attention.</td>
<td>164–69</td>
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<td>CRF OE (Dirks et al.)</td>
<td>Reduced acoustic startle reactivity, impaired pre-pulse inhibition, and abnormal behavioral adaptation to a novel environment.</td>
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<td>CRF OE (CNS restricted)</td>
<td>Stress-induced hyperscretion of ACTH and corticosterone (only in males). Reduced immobility in the forced swim test and tail suspension test.</td>
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<tr>
<td>CRF KO</td>
<td>Normal basal and stress-induced anxiety-like behavior, locomotor activity, exploration, startle response and learning.</td>
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<tr>
<td>CRFRI KO</td>
<td>Disrupted HPA axis activation in response to stress. Reduced anxiety-like behavior, impaired spatial recognition memory, and deficiencies in nurturing behavior (females).</td>
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<td>CRFRII KO (Bale et al.)</td>
<td>Increased ACTH and corticosterone response to stress and early termination of ACTH release. Increased anxiety and depression-like behavior.</td>
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<tr>
<td>CRFRII KO (Coste et al.)</td>
<td>Increased ACTH and corticosterone response to stress and early termination of ACTH release. No differences in anxiety-like behavior.</td>
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<tr>
<td>CRFRII KO (Kishimoto et al.)</td>
<td>Increased anxiety and depression-like behavior.</td>
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<td>CRF-BP OE</td>
<td>Increased locomotion and rearing in the open field test, and increased total arm entries in the EPM test.</td>
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<tr>
<td>CRF-BP KO</td>
<td>Increased anxiety-like behavior. Deficits in maternal aggression.</td>
<td>190, 191</td>
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<tr>
<td>CRFRI/CRFRII dKO</td>
<td>Impaired stress-induced HPA system activation. Reduced anxiety-like behavior in the EPM test (only in females).</td>
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<tr>
<td>CRFR1&lt;sup&gt;loxP/loxP&lt;/sup&gt;CamKIIzCRE</td>
<td>Reduced anxiety-like behavior. Normal basal plasma ACTH and corticosterone levels.</td>
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<tr>
<td>Ucn1 KO (Vetter et al.)</td>
<td>Increased anxiety-like behavior.</td>
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<td>Ucn1 KO (Wang et al.)</td>
<td>No effect in anxiety-like behavior.</td>
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<td>Ucn2 KO</td>
<td>Increased nocturnal ACTH and corticosterone levels, and reduced depressive-like behavior (only in females). No effect in anxiety-like behavior.</td>
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<tr>
<td>Ucn3 KO</td>
<td>Normal HPA axis regulation, anxiety-like behavior and depressive-like behavior. Increased social recognition memory.</td>
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<tr>
<td>Ucn1/Ucn2 dKO</td>
<td>Increased stress-induced corticosterone response (only in males). Decreased anxiety-like behavior.</td>
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<td>Ucn1/Ucn2/Ucn3 tKO</td>
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metallothionein (mMT1) promoter have been developed. CRF-overexpressing mice (CRF-OE) exhibit high plasma levels of ACTH and corticosterone, and develop physical changes typical of Cushing’s syndrome. At the behavioral level these mutants display a phenotype characteristic of increased anxiety and depression levels, such as decreased baseline and stress-induced locomotor activity in a novel environment, decreased time spent in the open arms of an elevated plus maze (EPM), decreased time spent in the light area of a light–dark transfer box, reduced time spent rearing in home cages, and increased immobility in a forced swim test. In terms of sexual behavior, CRF-OE female mice display a profound decrease in sexual receptivity, whereas the sexual performance of CRF-OE males remains intact. CRF-OE mice also show significant deficits in learning and reduced attention.

An additional CRF-OE transgenic mouse was generated using the murine Thy-1.2 promoter. In contrast to the mMT1 CRF-OE mice, the overexpression in Thy1 CRF-OE mice starts 4–8 days after birth and is restricted to the central nervous system and the spinal cord. These mice display reduced acoustic startle reactivity, impaired pre-pulse inhibition, and abnormal behavioral adaptation to a novel environment.

Finally, three different mouse models of conditional CRF overexpression have been generated using the nestin (Nes) promoter (CNS-restricted overexpression), CamKIIα promoter (forebrain-restricted overexpression) and Dlx5/6 promoter (forebrain GABAergic neurons-restricted overexpression). CRF overexpression in the entire central nervous system, but not in specific forebrain regions, resulted in stress-induced hypersecretion of ACTH and corticosterone in males but not in females, and reduced immobility in the forced swim test and tail suspension test in both males and females.

CRF-KO

The development of CRF knockout (CRF-KO) mice has been important in addressing the physiologic and pathologic roles of CRF. However, in terms of behavioral effects, CRF-KO mice exhibit normal basal and stress-induced anxiety-like behavior. In addition, baseline locomotor activity, exploration, startle response and learning appear to be unaffected. CRF receptor antagonists have an anxiolytic effect in CRF-KO mice, suggesting that whereas CRFR1 activation is crucial to induce anxiety, CRF itself is not. Therefore, it has been proposed that another CRF-related peptide, such as Ucn1, which has high affinity to CRFR1, may contribute to the observed stress-induced behavior in these animals.

CRFR1-KO

In order to elucidate the relative contribution of the CRFR1 receptor to stress-induced physiology and behavior, two independent groups generated a mouse line null for the CRFR1 gene. Mice deficient for CRFR1 (CRFR1-KO) display a disrupted HPA axis activation in response to stress, causing glucocorticoid deficiency. However, basal plasma ACTH was found to be similar to that in wild-type controls, probably due to the action of AVP. Behaviorally, CRFR1-KO mice display reduced anxiety-like behavior in the light–dark transfer test and the EPM test. In addition, it has been reported that CRFR1-KO mice are impaired in spatial recognition memory in a Y-maze paradigm. In terms of social behavior, male CRFR1-KO mice show intact isolation-induced inter-male aggression, whereas female CRFR1-KO mice exhibit deficiencies in nurturing behavior, such as decreased time spent nursing, licking and grooming the pups. It should be remarked that, in both studies, CRFR1-KO mice with C57BL/6 X 129 background, have been crossed into an outbred hsd:ICR strain selectively bred to exhibit high levels of maternal aggression, to improve aggressive performance.

CRFR2-KO

In contrast to the clear and consistent phenotype of the two CRFR1-KO mouse lines, the three independently generated CRFR2-KO mouse lines present several inconsistencies in their neuroendocrine and behavioral profiles. Coste and colleagues found an increased ACTH and corticosterone response to stress and an early termination of ACTH release in CRFR2-KO mice. However, no differences in anxiety-like behavior were found between CRFR2-KO and their wild-type littermates. Bale et al. also showed increased ACTH and corticosterone response to stress and an early termination of ACTH release in their CRFR2-KO mouse line. However in contrast to the Coste et al. findings, Bale and colleagues found that CRFR2-KO mice display a significant increase in anxiety-like behavior in the EPM and the open field tests, but not in the light–dark transfer test. Finally, Kishimoto et al. showed that only male CRFR2-KO mice are more anxious in the EPM test and the light–dark transfer test; however, in the open field test, CRFR2-KO mice spent more time in the center of the arena. These differences could be a result of the different genetic backgrounds of the mouse lines. Both the Bale et al.
and the Kishimoto et al. CRFR2-KO mice lines exhibited a depression-like phenotype in the forced swim test and the tail suspension test.\textsuperscript{186,187}

**CRF-BP-OE**

Two different mouse models of CRF binding protein overexpression (CRF-BP-OE) have been independently generated.\textsuperscript{188,189} The CRF-BP-OE line created by Lovejoy and colleagues expresses the transgene not only in the brain and pituitary, but also ectopically in peripheral tissues.\textsuperscript{189} However, this mouse line has not yet been behaviorally phenotyped. The CRF-BP-OE line created by Burrows et al. expresses the transgene under the control of the pituitary glycoprotein hormone \(\alpha\)-subunit (\(\alpha\)-GSU) promoter, resulting in specific overexpression of CRF-BP in the anterior pituitary gland.\textsuperscript{188} These mice exhibit increased locomotion and rearing in the open field test, increased total arm entries in the EPM, and a tendency to spend more time in the open arms of the EPM.\textsuperscript{188}

**CRF-BP-KO**

Mice deficient for CRF-BP (CRF-BP-KO) display increased anxiogenic-like behavior when tested in the EPM and the light–dark transfer test, and a trend toward increased anxiety-like behavior in the open field test.\textsuperscript{190} These results suggest an increase in “free” CRF or Ucn1 levels, leading to an anxiogenic phenotype; however, this hypothesis has not yet been examined in these mutants. The same line of CRF-BP-KO mice has been crossed into an outbred hsd:ICR strain selectively bred to exhibit high levels of maternal aggression, to investigate aggressive behavior.\textsuperscript{191} In this study, CRF-BP-KO mice exhibited significant deficits in maternal aggression (offspring protection) relative to wild-type mice. Female CRF-BP-KO mice were also tested in the forced swim test, where no differences were found between the groups. In the light–dark transfer test, female CRF-BP-KO mice exhibited higher levels of anxiety-like behavior. For males, no significant differences in light–dark transfer, swim test and isolation-induced resident–intruder male aggression were found between the groups. However, increased anxiety-like behavior in mutant males was detected in the approach to a novel object placed in the center of an open field arena.\textsuperscript{191}

**CRFR1/CRFR2 Double KO (dKO)**

To further elucidate the role of both CRF receptors in the activation and modulation of the neuroendocrine stress response and anxiety-like behavior, two mouse models deficient of both CRFR1 and CRFR2 (CRFR1/CRFR2 double knockout; dKO) have been independently generated.\textsuperscript{192,193} Both mouse lines display impairment in stress-induced HPA system activation. In terms of anxiety-like behavior, double-mutant mice are sexually dichotomous. Whereas no difference was found in anxiety-like behavior between male double-mutants and wild-type mice, female CRFR1/CRFR2 dKO mice display reduced anxiety-like behavior in the EPM test. However, no difference was found in the number of center visits in the open field test.\textsuperscript{192} The behavioral phenotype of the Preil et al. mouse model\textsuperscript{193} has not yet been reported.

**CRF1\textsuperscript{loxP/loxP}CamKII\(\alpha\)CRE**

As glucocorticoids are known to be involved in the modulation of fear and anxiety-like behavior,\textsuperscript{194,195} the anxiolytic-like profile observed in the developmental CRFR1 KO mice may result either from central CRFR1 deficiency, or from the low circulating levels of glucocorticoids. In order to differentiate the CRF/CRFR1 pathways that control stress-induced behavior from those regulating the HPA axis, a conditional CRFR1 knockout mouse line was generated using the Cre/loxP system. In these mutants the CRFR1 expression, driven by the calcium calmodulin-kinase II\(\alpha\) (CamKII\(\alpha\)) promoter, is postnatally ablated in the anterior forebrain and limbic system including the hippocampus, amygdala and neocortex, but remains intact in the pituitary gland.\textsuperscript{196} The CRFR1 conditional mutants (CRF1\textsuperscript{loxP/loxP}CamKII\(\alpha\)CRE) display reduced anxiety-like behavior levels in the EPM and the light–dark transfer tests. In contrast, the basal plasma ACTH and corticosterone levels are similar to those in wild-type mice. Moreover, the hormone levels in conditional mutants remain significantly elevated 30 and 90 min following restraint stress, indicating that CRFR1 conditional mutants are hypersensitive to stress, and that limbic CRFR1 is required for central control of HPA system feedback and hormonal adaptation to stress.\textsuperscript{196}

**Ucn1-KO**

Two mouse lines deficient for the Ucn1 gene (Ucn1-KO) were independently generated.\textsuperscript{197,198} Ucn1-KO mice show normal endocrine stress responses in both mouse lines,\textsuperscript{197,198} supporting the view that Ucn1 does not play a role (or has only a minor one) in stress-induced HPA axis regulation. Similar to the case of CRFR2-KO, the behavioral phenotype of Ucn1-KO mice is controversial. Whereas Wang and colleagues showed no differences in anxiety-like behavior,\textsuperscript{199} Vetter et al. demonstrated that Ucn1-KO mice have increased anxiety-like behavior in the EPM and the open field tests; however, no differences were found in the light–dark transfer test.\textsuperscript{197}
Ucn2-KO

Mice deficient for Ucn2 (Ucn2-KO) exhibit gender-specific alterations in the basal circadian rhythms of ACTH and corticosterone secretion, and in depressive-like behavior. The nocturnal ACTH and corticosterone levels were found to be higher in female Ucn2-KO mice compared to their wild-type littermates, but not in male. No differences were found in stress-induced hormone response in both genders. In addition, female mutant mice display reduced depressive-like behavior assessed by both the forced swim test and the tail suspension test. The altered performance of female Ucn2 null mice in tests of antidepressant activity was behaviorally specific, because Ucn2 null mutant mice did not differ in their anxiety-like behavior in the EPM or light–dark transfer tests. In addition, the differences observed in the forced swim test could not be attributed to deficits in learning abilities, as no differences were found between the genotypes in a cued contextual fear conditioning test.

Ucn3-KO

Mice deficient for the Ucn3 gene (Ucn3-KO) were independently generated by two groups. Li et al. revealed an important role for Ucn3 in the regulation of glucose-induced insulin secretion in the pancreas, while not reporting any behavioral phenotype. Deussing et al. recently assessed the second Ucn3 KO mouse model in several behavioral tests. No differences were found in HPA axis regulation, anxiety-like behavior or depressive-like behavior between the genotypes in a cued contextual fear conditioning test.

Ucn1/Ucn2 Double KO (dKO)

To further explore the physiological role of Ucn1 and Ucn2 in mediating the central stress response, a double Ucn1 and Ucn2-deficient mouse line (Ucn1/Ucn2 dKO) was recently generated. Ucn1/Ucn2 dKO male mice show a higher stress-induced corticosterone response and an anxiolytic profile in the EPM, the open field and the light–dark transfer tests, relative to wild-type mice. Female Ucn1/Ucn2 dKO mice show no significant changes in basal or stress-induced corticosterone levels. However, similar to male mutants, an anxiolytic-like phenotype was observed in the EPM and the open field tests. Whereas both female and male wild-type mice exhibited an expected increase in anxiety-like behavior when subjected to restraint stress before the tests, Ucn1/Ucn2 dKO mice showed a significantly smaller stress-induced change in behavior.

Ucn1/Ucn2/Ucn3 Triple KO (tKO)

To better understand the role of the endogenous CRF2 ligands Ucn1, Ucn2 and Ucn3 in regulating the central stress response, a triple knockout (tKO) mouse model lacking all three urocortin genes has been generated. Intriguingly, these urocortin tKO mice exhibit increased anxiety-like behaviors in the open field and light–dark transfer tests, 24 h following stress exposure, but not under unstressed conditions or immediately following exposure to acute stress. In addition, urocortin tKO mice exhibit a significantly higher stress-induced increase in acoustic startle-response. Together, these results suggest that lacking all urocortins has a limited effect on anxiety under non-challenged conditions but renders the mice susceptible to the effects of stress, possibly by impairing recovery mechanisms.

DYSREGULATION OF THE CRF SYSTEM: MOOD DISORDERS

Depression

Several human studies have suggested that abnormal CRF neurotransmission and CRFR1 receptor signal transduction play a central role in the pathophysiology of depression (reviewed in Holsboer and Ising, Binder and Nemeroff). For instance, the CSF concentrations of CRF are elevated in depressive patients as well as in suicide victims when compared to normal controls. In contrast, electroconvulsive therapy or antidepressant administration results in reduced levels of CRF in the CSF. Following CRF administration, depressive patients display blunted ACTH release, suggesting a desensitization of CRF receptors caused by CRF hypersecretion. Consistent with this hypothesis, it was found that CRF binding sites are reduced in the forebrain of depressive suicide victims, whereas CRF concentrations are elevated in the PVN, pontine nucleus, locus coeruleus and cortex. Moreover, in depressive patients Ucn1 is upregulated in the Edinger-Westphal nucleus and Ucn3 in the prefrontal cortex, suggesting a possible role for CRFR2 in the development of depression.

Several genetic association studies have linked polymorphisms in the CRF family genes with depression and suicidality. A CRFR1 haplotype with alleles G-A-G for the single nucleotide polymorphisms (SNP)
A few association studies have linked polymorphisms in the CRF family with anxiety disorders. In a large number of selective CRFR1 antagonists from different pharmaceutical companies have been developed in the past two decades as novel therapeutics for anxiety and depression. However, few of them have entered clinical development and, despite major efforts, to date no CRFR1 antagonist has successfully completed a Phase III trial. An open-label Phase IIa clinical trial assessed the effects of NBI-30775/R121919, a non-peptide high-affinity CRFR1 antagonist, in depressed patients. The group receiving NBI-30775/R121919 exhibited a safety and tolerability profile, increased slow-wave sleep and decreased REM density, and reduced depression and anxiety scores. Several other CRFR1 antagonists are currently in clinical development for the treatment of anxiety and depression.

A few association studies have linked polymorphisms in the CRF family with anxiety disorders. In addition, allele G carriers of the rs2270007 SNP in the CRFR2 gene showed a worse overall response to antidepressant treatment. Finally, one study found that the rs10473984 SNP within the CRFBP locus showed a significant association with both remission and reduction in depressive symptoms following antidepressant treatment.

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Concluding Remarks

Stress-related psychopathologies, such as depression and anxiety disorders, represent some of the most common and proliferating health problems worldwide. Better understanding of the etiology of these disorders may fill the urgent requirement for development of novel and improved therapeutics. The current view on the etiology of mental disorders is of a complex interaction between genetic and environmental factors. Certain genetic backgrounds might constitute a predisposition for the development of mental disorder, which will onset with the occurrence of additional environmental factors.

One of the key environmental risk factors that contribute to the onset of a variety of psychopathologies is stress. When a situation is perceived as stressful, the brain activates many neuronal circuits, linking centers involved in sensory, motor, autonomic, neuroendocrine, cognitive and emotional functions in order to adapt to the demand. However, the details of the pathways by which the brain translates stressful stimuli into the final, integrated biological response are not completely understood. Nevertheless, it is clear that dysregulation of these physiological responses to stress can have severe psychological and physiological consequences, and there is growing evidence to suggest that inappropriate regulation, disproportional intensity, or chronic and/or irreversible activation of the stress response is linked to the etiology and pathophysiology of anxiety disorders and depression.

Previous studies suggested that the CRF/urocortin systems in the brain have a unique role in mediating behavioral and physiological responses to diverse stimuli.
stressors.  However, while the involvement of the CRF/CRF1 system in regulating the activation of the HPA axis and the regulation of stress-linked behaviors is well established, the role of the central urocortins/CRF2 system is less understood. The urocortins/CRF2 system may be particularly important in situations where an organism must mobilize not only the HPA system but also the central nervous system in response to environmental challenge. Clearly, dysfunction in such a fundamental brain-activating system may be the key to a variety of pathophysiological conditions involving abnormal responses to stressors, such as anxiety disorders, affective disorders and anorexia nervosa.

Evidence from studies employing competitive peptides, or small-molecule CRF/urocortin receptor antagonists, suggested that the brain CRF/urocortin systems play diverse roles in mediating behavioral responses to stress. Based on the complementary behavioral phenotypes of CRF1- and CRF2-deficient (KO) mice, opposing roles were suggested for the two CRF receptor systems in modulating anxiety-like behaviors. CRF1-KO mice display decreased anxiety-like behaviors coupled with an impaired HPA axis stress response, while CRF2-KO mice show increased anxiety-like behaviors and an accelerated HPA axis response to stress. Thus, the CRF–CRF1 system was suggested as critical for initiating stress responses, while the urocortins–CRF2 system was suggested to terminate it or restore allostatic. Nevertheless, the anxiety-related effects of CRF2 antagonist and agonist administration into the cerebral ventricles or into specific brain regions were less consistent, with some evidence for brain-site or ligand specificity.

Mice lacking all three urocortin genes (tKO) exhibit increased anxiety-like behaviors 24 hours following stress exposure, but not under unstressed conditions or immediately following exposure to acute stress. This suggests that the urocortins might play an essential role in the stress-recovery process. Mouse models of Ucn1, Ucn2 and Ucn3 individual KOs have not indicated clear and robust changes in stress-related behaviors; this may reflect differences in the time point following the stress exposure at which these mice were tested. Therefore, further “dissection” of the contribution of each urocortin to the tKO phenotype using longitudinal comparative studies in both sexes, under different time points following stress exposure, may promote further understanding of the role of each urocortin gene product in the regulation of the central stress response.

Conventional gene knockout models, generated for the different urocortin genes, have provided important information toward elucidating the function of these genes. However, these mice showed significant changes in the expression levels of the other CRF family members in the CNS, likely due to developmental compensatory mechanisms, which may have contributed to the observed stress-related phenotypes. In order both to avoid the developmental compensatory changes and to genetically target the gene of interest within specific brain nuclei, future studies will need to use more specific transgenic mice models and viral tools that will allow the manipulation of both the levels and site of urocortin gene expression in adult mice.

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III. HORMONES, BRAIN FUNCTION AND BEHAVIOR


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