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Urocortins: CRF's siblings and their potential role in anxiety, depression and alcohol drinking behavior

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Abstract

It is widely accepted that stress, anxiety, depression and alcohol abuse-related disorders are in large part controlled by corticotropin-releasing factor (CRF) receptors. However, evidence is accumulating that some of the actions on these receptors are mediated not by CRF, but by a family of related Urocortin (Ucn) peptides Ucn1, Ucn2 and Ucn3. The initial narrow focus on CRF as the potential main player acting on CRF receptors appears outdated. Instead it is suggested that CRF and the individual Ucns act in a complementary and brain region-specific fashion to regulate anxiety-related behaviors and alcohol consumption. This review, based on a symposium held in 2011 at the research meeting on “Alcoholism and Stress” in Volterra, Italy, highlights recent evidence for regulation of these behaviors by Ucns. In studies on stress and anxiety, the roles of Ucns, and in particular Ucn1, appear more visible in experiments analyzing adaptation to stressors rather than testing basal anxiety states. Based on these studies, we propose that the contribution of Ucn1 to regulating mood follows a U-like pattern with both high and low activity of Ucn1 contributing to high anxiety states. In studies on alcohol use disorders, the CRF system appears to regulate not only dependence-induced drinking, but also binge drinking and even basal consumption of alcohol. While dependence-induced and binge drinking rely on the actions of CRF on CRFR1 receptors, alcohol consumption in models of these behaviors is inhibited by actions of Ucns on CRFR2. In contrast, alcohol preference is positively influenced by actions of Ucn1, which is capable of acting on both CRFR1 and CRFR2. Because of complex distribution of Ucns in the nervous system, advances in this field will critically depend on development of new tools allowing site-specific analyses of the roles of Ucns and CRF.

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Keywords

urocortin; corticotropin releasing hormone; alcoholism; anxiety; depression; addiction

Introduction

It is well known that the corticotropin-releasing factor (CRF, also known as the corticotropin-releasing hormone) peptide system is critical for the neuroendocrine and behavioral responses to stressful situations (such as anxiety and depression) in vertebrates (Bale and Vale, 2004; Hauger et al., 2006). Since stress is one of the risk factors of alcoholism, much evidence has been gained confirming the involvement of the CRF system in alcohol abuse and dependence (Heilig and Egli, 2006; Koob and Le Moal, 2001). However, the role of CRF system has been too often simplistically equaled with the role of CRF. This is not surprising, as historically CRF was the first peptide of the CRF system to be discovered (Vale et al., 1981).

It is now appreciated that the CRF system is more complex than previously thought and includes several additional players. Specifically, the CRF system includes, in addition to CRF, the three urocortin peptides (Ucn1, Ucn2 and Ucn3), two receptors types, CRFR1 and CRFR2 and the CRF-binding protein (Bale and Vale, 2004; Fekete and Zorrilla, 2006; Joels and Baram, 2009; Kuperman and Chen, 2008; Ryabinin et al., 2002; Steckler and Holsboer, 1999). Table 1 shows that Ucn3 binds and activates the CRFR2 with high affinity. CRF has a relatively lower affinity for CRFR2 than for CRFR1; Ucn1 has equal affinities for both receptors; and Ucn2 and Ucn3 appear to be selective for CRFR2 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001; Vaughan et al., 1995).

The CRF receptors are distributed differently throughout the brain: while CRFR1 is widely expressed, CRFR2 is expressed in a more discrete but partially overlapping manner. Selective expression of CRFR2 is observed in anxiety and depression-related brain nuclei, including the medial amygdala (MeA), bed nucleus of stria terminalis (BNST), lateral septum (LS) and the dorsal raphe nucleus (DRN) (Chalmers et al., 1995; Steckler and Holsboer, 1999; Van Pett et al., 2000). CRF peptide has been found in the paraventricular nucleus of hypothalamus (PVN), neocortex, central nucleus of amygdala (CeA), BNST, hippocampus, raphe nuclei, periaqueductal gray, olfactory bulbs, several thalamic and brain stem nuclei and the cerebellum (Merchenthaler et al., 1983; Morin et al., 1999; Steckler and Holsboer, 1999; Swanson et al., 1983). Ucn1 is primarily expressed in the centrally-projecting Edinger-Westphal nucleus (EWcp) (Bittencourt et al., 1999; Kozicz et al., 1998; Ryabinin et al., 2005; Vaughan et al., 1995). This brain region (also previously called non-preganglionic Edinger-Westphal nucleus and the perioculomotor urocortin-containing area) should be distinguished from the preganglionic Edinger-Westphal nucleus (EWpg), a cholinergic parasympathetic nucleus known for its oculomotor function, which does not contain Ucn1 (Cavani et al., 2003; Kozicz et al.; May et al., 2008; Ryabinin et al., 2005; Vasconcelos et al., 2003; Weitemier et al., 2005). Earlier literature did not distinguish between EWcp and EWpg, and most often referred to the site of Ucn1 as EW. Besides EWcp, the lateral superior olive and supraoptic nucleus express Ucn1, although at lower levels, and inconsistently between different species (Bittencourt et al., 1999; Spina et al., 2004; Weitemier et al., 2005). Ucn2 is expressed in the PVN, supraoptic nucleus, arcuate nucleus, locus coeruleus, the trigeminal, facial and hypoglossal motor nuclei and the meninges (Reyes et al., 2001; Tanaka et al., 2003). Ucn3 is expressed in medial preoptic area, perifornical area, BNST, MeA, ventral premammillary nucleus, superior olivary nucleus and parabrachial nucleus (Cavalcante et al., 2006; Deussing et al.; Lewis et al., 2001; Li et al., 2002). It also needs to be kept in mind that differences in the distribution of

these peptides and receptors between species and even lines of animals have been reported, further complicating the discussion of their function (Weitemier et al., 2005).

The pivotal role of CRF expressed in the PVN, acting on CRFR1 receptors in the pituitary and mediating the hypothalamic-pituitary-adrenal (HPA) axis response to stressors has been well established. Therefore, at first it appeared surprising that while both CRFR1 KO and CRF KO showed HPA deficits, deletion of CRFR1, but not CRF, lead to attenuation of anxiety-like behaviors (Weninger et al., 1999). This evidence suggested that other CRF receptor ligands (such as the Ucns) play important roles in the behavioral responses to stressors. Recent studies have focused on elucidating these roles using different methodologies and revealed the importance of Ucns in behaviors related to adaptation and mal-adaptation to stress, such as anxiety, depression and alcohol consumption. Importantly for alcohol research, these studies implicate the Ucns not only in dependence-induced drinking, but also in binge drinking of alcohol. This review focuses on the recent findings in this field.

Role of Urocortins in adaptation to stress and anxiety: genetic evidence

While the role of the CRF-CRFR1 system in activating the HPA axis and regulating emotional and cognitive functions following exposure to stressors is well established (Arborelius et al., 1999; Holsboer, 1999; Nemeroff, 1992; Reul and Holsboer, 2002), the role of the Ucns-CRFR2 system is only beginning to be understood. Interpretation of pharmacological studies testing the roles of specific peptides in stress and anxiety has been difficult because of the partially overlapping patterns of distribution of CRF, Ucns and both types of receptors. In contrast, studies using genetic KO of CRF receptors and their ligands have allowed distinction of some of these roles.

While CRFR1 KO mice clearly showed decreased measures of anxiety and stress (Smith et al., 1998; Timpl et al., 1998), CRFR2 KOs tended to show heightened anxiety (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000). Therefore, it was suggested that the CRF-CRFR1 system is essential for initiating stress responses whereas the Ucns-CRFR2 system is involved in terminating the stress response. While this hypothesis is not without contradictions, it has gained much support. However, the three independently generated CRFR2-KO mouse lines (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000) presented several inconsistencies in their endocrine and behavioral profiles, possibly due to differences in background lines. For example, Coste and colleagues reported an increased ACTH and CORT response to stress and an early termination of ACTH release in CRFR2-KO mice, but found no differences in anxiety-like behavior between CRFR2-KO and WT littermates (Coste et al., 2000). On the other hand, Bale and colleagues reported increased ACTH and CORT response to stress and an early termination of ACTH release in their CRFR2-KO mouse line (Bale et al., 2000). Yet, these CRFR2-KO mice exhibited a significant increase in anxiety-like behavior in the elevated plus maze (EPM) and the open field (OF), but not in the dark-light transfer (DLT), tests. In partial agreement with these phenotypes, Kishimoto and colleagues reported that CRFR2-KO male mice appear more anxious in the EPM and DLT tests, although in the OF test they spent more time in the center area, indicative of anxiolysis (Kishimoto et al., 2000). Interestingly, the latter two lines also exhibited a depression-like phenotype in the forced swim (FS) and tail suspension (TS) tests (Bale and Vale, 2003; Todorovic et al., 2009). In addition, CRFR2-KO females have been shown to have impaired maternal defense behavior of their offspring (Gammie et al., 2005) and enhanced social discrimination memory (Deussing et al.).

The first two independently generated mouse *Ucn1*-KO models were reported to exhibit normal endocrine stress responses (Vetter et al., 2002; Wang et al., 2002), supporting the

view that Ucn1 has a minor role in stress-induced HPA axis regulation. Like CRFR2-KO mice, their behavioral phenotypes were inconsistent. Whereas Wang and colleagues (Wang et al., 2002) reported no differences in anxiety-like behaviors, Vetter and colleagues (Vetter et al., 2002) reported increased anxiety-like behaviors in the EPM and OF, but not in the DLT, tests. In a more recently developed Ucn1-KO line a slightly increased glucocorticoid response to an intermittent restraint stress in male (but not female) mice was reported (Zalutskaya et al., 2007).

Ucn2-KO mice have been shown to exhibit sex-specific alterations in the basal circadian rhythms of ACTH and corticosterone secretion, and in depression-like behavior (Chen et al., 2006). In comparison with WT littermates, nocturnal ACTH and CORT levels were elevated in KO-females, but not KO-males. No differences were found in stress-induced hormone response in either sex. Furthermore, female Ucn2-KOs displayed reduced depression-like behavior in the FS and TS tests. Yet female Ucn2-KO mice did not differ from WTs in anxiety indices in the EPM or DLT tests, or in cued and contextual fear conditioning tests (Chen et al., 2006).

Ucn3-KO mice were independently generated by two groups (Deussing et al.; Li et al., 2007). Li and colleagues reported a role for Ucn3 in the regulation of glucose-induced insulin secretion in the pancreas, but did not test them behaviorally (Li et al., 2007). Deussing and colleagues (2010) recently reported no differences between Ucn3-KO mice and WT littermates in HPA axis regulation, anxiety-like or depression-like behaviors (EPM, FS, acoustic startle reflex and fear conditioning). However, in a social discrimination task, Ucn3-KOs recognized previously encountered conspecifics after a longer time than WTs (a similar observation to CRFR2 KOs), suggesting a more specific role in social behaviors for this peptide (Deussing et al.).

A unique double Ucn1 and Ucn2 deficient mouse line (Ucn1/Ucn2 dKO) was recently generated in Dr. Alon Chen's laboratory (Neufeld-Cohen et al.). In comparison with WT mice, Ucn1/Ucn2 dKO males exhibited an increased stress-induced CORT response and an anxiolytic profile in the EPM, OF and DLT tests. Female Ucn1/Ucn2 dKO mice did not differ from WTs in basal or stress-induced CORT levels, yet, as in males, an anxiolytic-like phenotype was evident in the EPM and OF tests. Interestingly, both male and female Ucn1/Ucn2 dKO mice exhibited a significantly attenuated stress-induced increase in anxiety indices in those tests (Neufeld-Cohen et al.).

A novel triple-knockout mouse model, lacking all three urocortin genes (Ucn-tKO) was also recently generated to further the understanding of the role of the endogenous CRFR2 ligands in regulating the central stress response (Neufeld-Cohen et al.). In comparison with WT mice, Ucn-tKO mice exhibited increased anxiety-like behaviors in the OF and DLT tests only when tested 24 hrs following acute stress exposure, but not under unstressed conditions or immediately following exposure to the stressor. In addition, Ucn-tKO mice exhibited a significantly higher stress-induced increase in acoustic startle-response. The inability of these KOs to recover properly from the exposure to an acute stress was associated with robust decreases in the expression of CRFR1, dopamine receptors DRD1a and DRD2, opioid receptors κ and μ , and the GABAergic synthesizing enzyme GAD and robust increase in expression of serotonin receptor 5HT_{3A} in amygdala. All of these genes are known players in stress responses, anxiety and mood. Furthermore, these Ucn-tKOs exhibited a dysregulated serotonergic function in amygdalar nuclei and within sub-regions of the septo-hippocampal system. The authors suggested that lacking all Ucn's 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 (Neufeld-Cohen et al.).

It is also noteworthy that studies exploring the role of the Ucn1-CRFR2 system using central administration of Ucn2 or Ucn3, CRFR2 antisense oligonucleotide (Heinrichs et al., 1997; Liebsch et al., 1999) and antagonists reported contrasting, dose-dependent and loci-dependent results, suggesting that this system's role varies across brain nuclei depending on different stress conditions (see (Sztainberg and Chen, 2011) for review). This is in agreement with the fact that Ucn1, Ucn2 and Ucn3 individual KO mouse models have not indicated a clear anxiety phenotype, perhaps because of differences in the time points of assessment following the stress exposure. Since the Ucn1-CRFR2 system was suggested to mediate restoration of homeostasis after stress (Coste et al., 2000; de Kloet et al., 2005; Joels and Baram, 2009) further testing individual Ucn KO models at time points that better reflect recovery processes, combined with the use of site-specific manipulations of those genes in adult mice (to avoid developmental compensatory changes), may promote further understanding of the role of each Ucn gene product in regulating the central stress response.

Ucn 1 and the moody brain

Since Ucn1 was the first discovered peptide among the mammalian Ucn1s (Vaughan et al., 1995), there has been more attention on Ucn1 than on Ucn2 or Ucn3. Soon after the discovery of Ucn1 in EWcp, it became clear that EWcp-Ucn1 neurons show robust activity changes in response to various acute behavioral and pharmacological manipulations (Bachtell et al., 2002a; Chang et al., 1995; Gaszner et al., 2004; Kozicz, 2007, 2009; Kozicz and A, 2001; Lanteri-Minet et al., 1993; Palkovits et al., 2009; Rouwette et al.; Spangler et al., 2009; Sterrenburg et al.; Weninger et al., 2000). Interestingly, activation of EWcp neurons and upregulation of Ucn1 mRNA and protein following pain stress often lasted more than 16 hours (Kozicz and A, 2001; Palkovits et al., 2009; Rouwette et al.), in contrast to the relative short-lasting (up to 2–4 hours) activation of PVN-CRF neurons (Viau and Sawchenko, 2002). These data support the notion that EWcp Ucn1 neurons are rather involved in the later, adaptive phase of the stress response (Kozicz, 2007; Kozicz and Sterrenburg, 2011; Rouwette et al.). This is in line with the idea that CRFR1 activated by CRF mediates the initial reaction to stress, whereas CRFR2 activated by Ucn1 controls the later adaptive phase, and that a balanced activation of CRFR1 and CRFR2 is crucial for homeostatic equilibrium (de Kloet et al., 2005; Joels and Baram, 2009; Kozicz, 2007; Kozicz and Sterrenburg, 2011; Reul and Holsboer, 2002).

Chronic exposure to stressors also recruits Ucn1 neurons in the rodent EWcp, as demonstrated by Fos immunoreactivity (Korosi et al., 2005; Xu et al.). These studies suggest that EWcp neurons do not habituate to chronic stressors, but rather stay active for a prolonged period of time (up to three weeks after stress initiation; (Korosi et al., 2005). This prolonged activation is in clear contrast to the fast habituating response of CRF-PVN neurons upon chronic stress (Viau and Sawchenko, 2002). In some chronic stress models, Ucn1 mRNA and protein levels in EWcp are also elevated for many days (up to one week), eventually returning to baseline after three weeks of chronic challenges (Korosi et al., 2005).

Taken together, the picture is emerging that stress mediators of the CRF/Ucn1-system operate in a well-orchestrated symphony (Bale and Vale, 2004; de Kloet et al., 2005; Joels and Baram, 2009; Kozicz and Sterrenburg, 2011; Reul and Holsboer, 2002), and that the complementary actions of CRF/CRFR1 and Ucn1/CRFR2 are crucial for body and mental health. We propose that Ucn1, mainly expressed in the EWcp, is important for maintaining a balanced activation of CRFR1 and CRFR2 during stress (Kozicz, 2007, 2009; Kozicz and Sterrenburg, 2011). Thus, while the functions on Ucn2 and Ucn3 could be to attenuate the response to the stressor, Ucn1 could be in charge of producing an adequate response. Consequently, this idea implies that EWcp-Ucn1 neurons may have a role in controlling mood (Kozicz, 2007, 2009; Kozicz and Sterrenburg, 2011).

Indeed, increasing evidence indicates specific roles for EWcp-Ucn1 neurons in anxiety-like behaviors. More specifically, peripheral and central administration of benzodiazepines and selective agonists of the metabotropic glutamate receptors recruit the EWcp neurons, suggesting that they may act through EWcp neurons (Bachtell et al., 2002a; Cespedes et al.; Linden et al., 2006; Linden et al., 2004; Skelton et al., 2000).

Furthermore, data from animals with genetic modification of the *Ucn1* gene described in the previous chapter for the most part substantiate the involvement of Ucn1 in regulation of anxiety-related behaviors. Together with the results in Ucn1-KOs showing increased anxiety-like behaviors 24 h following stress exposure, but not under unstressed conditions or immediately following exposure to acute stress (Neufeld-Cohen et al.), they support the idea that Ucn1 is one of the peptides that plays a more prominent role in the later adaptive phase of the stress response, and that the effect(s) of Ucn1 deficiency on mood will only be apparent in this later adaptive phase. As further evidence for the possible role of Ucn1 in controlling mood is the fact that male depressed suicide victims show an approximately ten times higher Ucn1 mRNA expression in EWcp compared to controls (Kozicz et al., 2008).

However, the fact that both the lack (*Ucn1* deficiency) as well as excessive amounts of Ucn1 (human post-mortem studies on depressed suicide victims) results in anxiety- and depression-like behavior is puzzling. To reconcile this, here we propose that the level of Ucn1 expression and the manifestation of anxiety- depression-like behavior are coupled by an inverted U shaped curve. Both low and high levels of Ucn1 may result in heightened anxiety- and depression-like behavior. Mechanistically, in Ucn1-KO mice, stress-induced release of CRF will not be counterbalanced by Ucn1 leading to an overactivation of CRFR1, and consequently increased anxiety- and depression-like behavior. Excessive amounts of Ucn1, as seen in depressed individuals, will not only activate CRFR2, but most probably will also recruit CRFR1. Concomitant with the hypersecretion of CRF as seen in depression (Arato et al., 1989; Arborelius et al., 1999; de Kloet et al., 2005; Hauger et al., 2006; Holsboer et al., 1984; Joels and Baram, 2009; Nemeroff, 1988) this would also lead to a strong overactivation of brain CRFR1 receptor signaling. Hypothetically, in both scenarios, the resulting imbalance in CRFR1/CRFR2 signaling in brain regions controlling mood will threaten homeostatic equilibrium and will enhance vulnerability for anxiety and depression. This intriguing hypothesis is currently under testing in the laboratory of T. Kozicz.

The role of corticotropin releasing factor system in binge-like ethanol intake: Pharmacological evidence

Previous preclinical investigations have demonstrated that both the CRFR1 (Chu et al., 2007; Funk et al., 2007; Gehlert et al., 2007; Hansson et al., 2006; Sommer et al., 2008) and the CRFR2 (Funk and Koob, 2007) in extrahypothalamic brain regions are critically involved in excessive dependence-like ethanol intake in rats stemming from exposure to ethanol vapor (Heilig and Koob, 2007; Lowery and Thiele, 2009). These studies have implicated peptides acting on CRF receptors and have been thoroughly reviewed (Cowen and Lawrence, 2006; Heilig and Koob, 2007; Koob). However, more recent studies have implicated CRF not only in dependence-induced excessive alcohol intake, but also in drinking in non-dependent animals. In particular pharmacological evidence indicates that this system is involved in binge alcohol drinking.

Binge drinking, defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as an episode of drinking that leads to blood ethanol concentrations (BECs) of 80 mg/dl or greater (Council, 2004), is a common behavior among adults and adolescents (Blazer and Wu, 2009; Courtney and Polich, 2009; Enoch, 2006) that increases the risk of developing ethanol dependence (Courtney and Polich, 2009). Binge drinking can be

modeled preclinically using the drinking-in-the-dark procedure (DID). This model is based on the notion that mice are nocturnal animals, and consume more fluid during the dark phase of the circadian cycle. While several modifications of this protocol have been developed (Grahame and Grose, 2003; Rhodes et al., 2005; Sharpe et al., 2005b; Thiele et al., 2003), the most commonly used is a 4-day protocol involving granting C57BL/6J mice limited access to 20% ethanol 3 hours into the dark portion of their light/dark cycle (Rhodes et al., 2005). Under these conditions, mice typically consume enough ethanol to achieve BECs well above 80 mg/dl, which leads to significant deficits in motor performance indicative of intoxication (Rhodes et al., 2005; Rhodes et al., 2007).

Given the clinical evidence that links binge drinking to an increased risk of ethanol dependence, and preclinical evidence that shows a role for CRF receptors in excessive dependence-like ethanol drinking, Dr. Todd Thiele's laboratory initially investigated the role of CRF receptor signaling in binge-like ethanol consumption. To this end, this laboratory assessed the effects of the peripherally bioavailable CRFR1 antagonist CP-154,526 (CP) on binge-like ethanol consumption by C57BL/6J mice using DID procedures (Sparta et al., 2008). Results showed that mice pretreated with a 10 mg/kg intraperitoneal (i.p.) injection of CP consumed significantly less ethanol and achieved significantly lower BECs than animals pretreated with vehicle (which achieved BECs in excess of 80 mg/dl). Importantly, the 10 mg/kg dose of CP did not alter consumption of a 10% sucrose solution. Interestingly, using a similar alternate ethanol access procedure that generated low, non-binge-like ethanol intake (with associated BECs of <40 mg/dl), the 10 mg/kg dose of CP did not alter ethanol consumption. Thus, CRFR1 signaling appears to be triggered when sufficient blood/brain ethanol levels are achieved, which may motivate continued binge-like ethanol drinking. These results parallel observations showing the CRF receptor antagonists blunt excessive dependence-like ethanol drinking in rats exposed to ethanol vapor but fail to influence low level ethanol intake by non-dependent rats (Funk et al., 2007). As such, CRF receptors appear to be recruited during excessive ethanol intake prior to the development of dependence, and it is speculated that plastic changes in CRF receptor signaling develop with repeated binge-like drinking episodes, contributing to the transition to dependence.

Subsequent investigations from Thiele's laboratory have revealed that central CRF receptor signaling is necessary for binge-like ethanol consumption, as mice pretreated with the CRF receptor antagonist α -helical CRF₉₋₄₁ in an i.c.v. infusion showed significant reductions of binge-like ethanol consumption relative to vehicle-treated mice. The antagonist α -helical CRF₉₋₄₁ failed to alter consumption of a 10% sucrose solution (Lowery et al.). Additionally, the role of CRF receptors in binge-like ethanol consumption appears to be primarily modulated by extrahypothalamic brain regions. Pretreatment with CP attenuated binge-like ethanol consumption in animals with impaired HPA axis signaling due to adrenalectomy. Furthermore, i.p. injection of the corticosterone synthesis inhibitor metyrapone or the glucocorticoid receptor antagonist mifepristone both failed to alter binge-like ethanol drinking, and binge-like ethanol drinking was not associated with alterations of circulating corticosterone levels, additional evidence that hypothalamic CRF receptor signaling is not involved in modulating binge-like ethanol intake (Lowery et al.). As there is evidence to suggest that CRFR2 signaling opposes the behavioral effects of the CRFR1 (Joels and Baram, 2009; Reul and Holsboer, 2002; Venihaki et al., 2004), the same study assessed the effects of the CRFR2 agonist Ucn3 on binge-like ethanol consumption. When given in an i.c.v. infusion, Ucn3 dose-dependently blunted binge-like ethanol intake and associated BECs by C57BL/6J mice (Lowery et al.). In addition to implicating a role for the CRFR2, these observations suggest a potential role for Ucn signaling in the modulation of binge-like ethanol intake. In summary, these pharmacological studies have provided evidence showing that blockade of the CRFR1 and activation of the CRFR2 protect against binge-like drinking, and that the effects of CRF receptor signaling on binge-like drinking appear to

involve extrahypothalamic sites. This is reminiscent of the roles of CRFR1 and CRFR2 found earlier in dependence studies, and suggest that CRF-like peptides are similarly involved in excessive alcohol drinking in dependent and non-dependent subjects. The present observations suggest that compounds aimed at CRF receptors may have therapeutic value for treating excessive binge drinking, perhaps protecting vulnerable individuals from progressing to dependence.

Differential roles of Ucns and CRF in regulation of alcohol intake

Since the pharmacological studies described above implicated both CRFR1 and CRFR2 in regulation of alcohol intake in dependent and non-dependent animals, and since CRF has low affinity to CRFR2 receptors (Bale and Vale, 2004), it was hypothesized that not only CRF, but also Ucns could be involved in this behavior (Ryabinin and Weitemier, 2006).

The first line of evidence in agreement with this possibility came from studies mapping immunoreactivity (IR) of the inducible transcription factors (ITFs) in brain. Initial studies mapped ITF IR following involuntary modes of alcohol administration in animals (i.e., intraperitoneal injections, vapor inhalation or intragastric intubation) (Chang et al., 1995; Knapp et al., 2001; Ogilvie et al., 1998; Ryabinin et al., 1997; Ryabinin et al., 1995; Thiele et al., 1996; Thiele et al., 1997). These studies showed a significant increase in IR of the ITF FOS in several brain regions, especially strong in CeA and EWcp, and suppression of FOS in hippocampal subregions. However, since exposure to novelty or aversive situations also induces FOS in many brain regions, it was difficult to determine FOS activation after alcohol administration was not due to the sudden stress associated with an unanticipated involuntary alcohol administration. In contrast, studies mapping ITF immunoreactivity following voluntary alcohol self-administration have produced unexpected results. They showed that while various paradigms used to train animals to self-administer relatively large amounts of alcohol in short amounts of time result in slightly different patterns of ITF IR, only one brain region, the EW, shows a consistent, robust and dose-dependent increase in FOS IR (Bachtell et al., 1999; Ryabinin et al., 2001; Toppo et al., 1988; Weitemier et al., 2001). This consistent effect has been demonstrated across different species (rats, mice and voles), genetic strains of animals and different modes of ethanol self-administration (dark and light circadian phase drinking, operant self-administration, unsweetened ethanol or ethanol supplemented with sucrose or beer) (Anacker et al.; Bachtell et al., 2003; Ryabinin et al., 2003; Sharpe et al., 2005b; Toppo et al., 1988; Weitemier et al., 2001). Importantly, the EW showing ethanol-induced increased FOS induction has been identified as EWcp, the main source of Ucn1 in the brain, and this response has been shown to be independent from EWcp FOS response to stress (Bachtell et al., 2002a; Bachtell et al., 2003; Ryabinin et al., 2003; Spangler et al., 2009; Turek and Ryabinin, 2005).

In order to further confirm the potential contribution of Ucn1 and EWcp, the laboratory of Dr. Andrey E. Ryabinin has performed a series of genetic studies analyzing levels of Ucn1 immunoreactivity in rodent strains differing in their predisposition to consume alcohol. Initial studies from this laboratory have shown that the inbred high alcohol-preferring C57BL/6J (B6) mice have significantly higher number of Ucn1-containing cells in EWcp than the inbred alcohol-avoiding DBA/2J (D2) mice (Bachtell et al., 2002b). This difference was shown to be due to both higher number of EWcp neurons and higher levels of Ucn1 IR in B6 versus D2 mice (Bachtell et al., 2002b; Weitemier and Ryabinin, 2005a). A difference in a molecular marker between two inbred strains of mice could be simply due to a random fixation of alleles. Therefore, these studies in inbred strains were followed up by analysis of various rodent strains selectively bred to differ in alcohol preference and intake. Studies in mice selectively-bred to prefer alcohol have shown that the high alcohol preferring lines (HAP-1 and HAP-2) had significantly higher levels of Ucn1 in EWcp than low alcohol preferring lines LAP-1 and LAP-2 (Bachtell et al., 2003). Similarly, HPP mice selectively

bred for high ethanol-induced conditioned place preference showed significantly higher levels of Ucn1 IR in EWcp than LPP mice selectively bred for low ethanol-induced conditioned place preference (Kiianmaa et al., 2003). The Ryabinin laboratory has also analyzed 7 lines of rats selectively bred for high alcohol intake and compared them to their corresponding genetic controls (Fonareva et al., 2009; Turek et al., 2005). Four lines, HAD2, msP, scr:Sp and HARF, showed significantly higher levels of Ucn1 in EWcp versus corresponding alcohol-avoiding, LAD2, Wistar (m), Wistar (Scr) and LARF lines. Two pairs of lines, the so called AA/NA and HAD1/LAD1 rats, showed no significant difference in Ucn1 IR between preferring and avoiding lines. One line, iP rats, showed significantly lower Ucn1 IR in EWcp than the corresponding iNP control line. While these data showed that differences in Ucn1 levels are not a necessary factor for differences in alcohol consumption, the fact that higher Ucn1 levels in preferring than in non-preferring animals occur in more than half of the tested pairs of selectively-bred rat lines and in all tested pairs of selectively-bred mouse lines is remarkable, and suggests that Ucn1 plays an important role in predisposition to high alcohol consumption.

To further elucidate this role, electrolytic lesions of EWcp have been developed. Studies using this approach showed that lesions of EWcp block alcohol intake and preference in B6 mice (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b). Remarkably, this effect is ethanol-specific, such that preference of other palatable fluids (i.e. sucrose or saline) or avoidance of bitter-tasting quinine, are not affected by the lesions. Interestingly, these lesions affect preference for 6 and 10% ethanol, alcohol solutions which B6 mice prefer, but do not affect preference of 20%, a solution that they do not prefer, suggesting that EWcp is involved in choosing the preferred ethanol solution.

One of the forebrain projections of EWcp is the lateral septum (Bachtell et al., 2004). Lateral septum is an area which frequently shows changes in activity in alcohol self-administration studies (Porrino et al., 1998; Ryabinin et al., 2003; Sharpe et al., 2005; Smith et al., 2001). Differences in Ucn1 immunoreactive-fibers have been identified between alcohol preferring and non-preferring rodent lines (Bachtell et al., 2003; Turek et al., 2005). Attempting to identify the neuroanatomical targets through which Ucn1 neurons of EWcp regulate alcohol drinking, pharmacological experiments using intracranial injections into the lateral septum of B6 mice have been performed. These experiments showed that a lower dose of Ucn1 than CRF was needed to produce an ethanol-specific decrease in intake, suggesting that this effect is most likely mediated by CRFR2, and not CRFR1 (Ryabinin et al., 2008). While these experiments confirmed that Ucn1 can regulate alcohol intake, they indicated that the facilitating effects of Ucn1 on alcohol intake revealed in the studies with selectively-bred rodents and EWcp lesions and described above are most likely not mediated through the lateral septum. In agreement with inhibitory effects of Ucn1 on alcohol intake, Sharpe et al. reported that Ucn3 injected i.c.v. decreased consumption of 10% ethanol in a limited-access procedure similar to the DID model of binge drinking (Sharpe and Phillips, 2009). This study was performed using a lickometer system allowing the researchers to conclude that the decrease in intake was due to a decrease in the size of the largest bout of drinking.

A number of studies have tested the involvement of specific components of the CRF system using KO approaches. They have revealed that peptides acting on CRFR1 and CRFR2 receptor regulate alcohol drinking differentially. In the 24-hr 2-bottle choice procedure, CRFR1 KO mice have been shown to consume ethanol intakes similar to WT mice when exposed to low (up to 10%) concentrations of ethanol (Chu et al., 2007; Pastor et al.; Sillaber et al., 2002). In contrast, consumption of 20% was reduced in these KOs (Pastor et al.). Prolonged intake in combination with repeated stress was shown to increase ethanol intake in CRFR1 KOs in one study (Sillaber et al., 2002), and decrease ethanol intake in

another (Pastor et al.). This discrepancy was hypothesized to be due to differences in genetic backgrounds of mice leading to different alcohol intake levels (Pastor et al.). On the other hand, exposure to ethanol vapor - an ethanol dependence inducing procedure - failed to produce an increase ethanol intake in CRFR1 KOs in contrast to their WT littermates, which increased intake. These KOs have also been recently shown to have decreased alcohol intake in the DID model of binge alcohol consumption (Kaur et al., in press). This decrease was specific to ethanol as CRFR1 KOs consumed larger volumes of sucrose than their WT littermates.

While alcohol intake in a 24-hour 2-bottle choice procedure and the DID procedure was not affected in CRFR2 KO mice, intake of 7.5% and 10% ethanol was significantly higher in CRFR2 KO versus WT littermates in some of the sessions in a study using 30-minute limited access procedure (Sharpe et al., 2005a).

Twenty four-hour 2-bottle choice alcohol consumption has been at first reported to be higher in the CRF KO mice compared to WT mice (Olive et al., 2003). However, rather than using WT littermate controls, these studies used control mice only approximately matching the genetic background of the KOs, and these control mice consumed low amounts of alcohol. In contrast, in a recent study comparing CRF KOs with WT littermates (an appropriate genetic control), alcohol consumption in the DID procedure was significantly lower in KO versus WT animals (Kaur et al., in press). Together with the pharmacological studies described in the previous chapter, and experiments showing decreased alcohol intake in CRFR1 KO mice, these results indicate that CRF acting on CRFR1 receptors is indeed one of the mechanisms critical for alcohol consumption in non-dependent animals.

Alcohol intake in Ucn1 KOs was tested in parallel with CRF KOs using the DID procedure (Kaur et al., in press). While no decrease in alcohol intake was found in Ucn1 KOs compared to WT animals, it was possible that the effect of Ucn1 on alcohol intake was masked by developmental compensations. Thus, early studies using these KOs have reported that as a result of developmental compensations, CRFR2 expression is decreased in the lateral septum of Ucn1 KO mice compared to WT mice (Vetter et al., 2002). To further address the possibility that developmental compensations could disrupt regulation of alcohol intake in Ucn1 KOs, the Ryabinin laboratory tested Ucn1 KO mice for c-Fos expression in EWcp following the DID procedure (Kaur et al., in press). Similar to previous studies (Bachtell et al., 1999; Sharpe et al., 2005b; Weitemier et al., 2001), blood ethanol concentrations (BECs) resulting from alcohol intake in WT mice were significantly correlated with the number of c-Fos-positive cells in EWcp ($r=0.76$, $p=0.0002$, $N=18$). In contrast, in the Ucn1 KOs consuming similar doses of ethanol, there was no significant correlation between BECs and the number of c-Fos-positive cells in EWcp ($r=0.27$, $p=0.29$, $N=17$). This difference in these correlations was statistically significant ($p=0.0005$; p -value calculated according to (Fisher, 1921; Wuensch et al., 2002)). These results are in agreement with the interpretation that developmental compensations resulting from deletion of Ucn1 disrupt the normal process of ethanol-induced neural activity within EWcp.

An alternative interpretation is that Ucn1 is not critical for regulation of binge-like drinking in the DID model, and that it is more involved in regulation of alcohol preference. In fact, the evidence for involvement of Ucn1 in alcohol drinking behavior discussed earlier in this section primarily involved alcohol preference models. A very recent study in the Ryabinin laboratory tested the combined effects of EWcp lesion and Ucn1 KO on alcohol intake in the continuous 2-bottle choice procedure (Giardino et al., in press). These study showed that deletion of Ucn1 and lesions of EWcp both decreased alcohol preference. These effects were dependent on each other (such that there was no further attenuation of alcohol preference by

EWcp lesion in Ucn1 KO mice), indicating that the effects of EWcp of alcohol preference were mediated by Ucn1.

Interestingly, the data on importance Ucn1 involvement in regulation of alcohol preference contrasts with lack of CRF involvement in regulation of drinking in non-dependent animals (Chu et al., 2007; Funk et al., 2007; Gehlert et al., 2007; Hansson et al., 2006; Sommer et al., 2008). Specifically, Ucn1 and CRF could be regulating different modes of alcohol drinking behavior: Ucn1 contributes to alcohol preference, whereas CRF contributes to binge drinking and dependence. This notion suggests that Ucn1 is involved in rewarding aspects of alcohol. To further test this idea, Ucn1 KO mice were tested in the ethanol-conditioned place preference and avoidance procedures. In agreement with the importance of Ucn1 for ethanol reward, Ucn1 KOs showed a lack of ethanol-induced conditioned place preference compared to WT littermates. No effects of Ucn1 deletion on ethanol-induced conditioned place aversion were found. The lack of ethanol-induced conditioned place preference was also found in CRFR2 KO mice, indicating that Ucn1 promotes rewarding aspects of ethanol through CRFR2 (Giardino et al., in press).

General conclusions and future directions

There has been much progress showing the contribution of Ucns to stress adaptation and regulation of alcohol consumption. The initial focus on CRF as the potentially main player acting on CRF receptors in regulation of these behaviors appears outdated. The importance of Ucns, and in particular Ucn1, in responses to stress and anxiety are more evident in experiments analyzing adaptation to repeated stressors than in experiments testing basal anxiety states or responses to acute stressors. This contribution of Ucns to these states appears bi-phasic. For example, we propose that the contribution of Ucn1 to regulation of mood could follow a U-like pattern with both high and low activity of Ucn1 contributing to high anxiety states. The CRF system appears to regulate not only dependence-induced drinking, but also binge drinking and perhaps even basal consumption of alcohol. While dependence and binge drinking depend on the actions of CRF on CRFR1 receptors, they are inhibited by actions of Ucns on CRFR2. In contrast, alcohol preference and low levels of alcohol consumption are positively influenced by actions of Ucn1 potentially acting on both CRFR1 and CRFR2. More precise mechanistic understanding of the roles of these peptides is complicated by complex brain region-specific distribution of CRF and Ucns, and probable different functions of these peptides in different brain regions. Novel site-specific transgenic and viral approaches are being developed to differentiate these functions. Advances in this field will critically depend on development of these tools.

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Table 1

Affinities of CRF-like peptides.

	CRF	Urocortin 1	Urocortin 2	Urocortin 3
CRFR1	Very high	Very high	Low	Low
CRFR2	Low	High-very high (species-dependent)	Very high	High
CRF-binding protein	Very high	Very high	Low-high (species-dependent)	Low

Affinities: Very high – inhibitory binding constants below 1 nM, High – inhibitory binding constants 1–10 nM, Low – inhibitory binding constants higher than 10 nM. The affinities are based on data in (Fekete and Zorilla, 2006).