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The Effects of Blocking Corticotropin-Releasing Hormone on Hypocretin-1-Induced Impairment of Maternal Behavior and Defense in Primiparous Mice

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Abstract

Environmental stressors can have a negative impact on maternal behavior. This likely occurs through excess peptides related to stress and arousal. Two of these potentially stress and arousal-related peptides are corticotropin-releasing hormone (CRH) and hypocretin (HCRT). Both CRH and HCRT induce anxiogenic behaviors in animals, and there is much evidence suggesting the peptides facilitate one another. HCRT and CRH also influence maternal behavior in similar ways. Both facilitate maternal behavior at moderate increases from baseline and impair behavior at higher levels. However, due to the bidirectional effects of these two peptides, it is unknown if these peptides affect maternal behavior by direct or indirect effects. In order to investigate how CRH and HCRT work together, this study examined the effects of blocking CRH receptors on maternal behavior after receiving a dose of HCRT-1 (0.3µg) that has previously been shown to decrease pup-directed behaviors and maternal defense. We hypothesized that blocking CRH receptors would rescue these behaviors. On three consecutive days, primiparous dams received counterbalanced intracerebroventricular (i.c.v.) injections of either a CRH antagonist at a low (2.5µg) or high (5µg) dose or a vehicle injection into the lateral ventricles, and then received HCRT-1 at 0.3µg. There were no treatment differences on pup directed behaviors. However, there were dose-dependent decreases in maternal defense observed at the high dose versus low dose of CRH antagonist, such that high dose decreased frequency of attacks and tail rattling. To explore neural activation in these dams following injection of HCRT-1 (0.3µg) or vehicle, immunohistochemistry was used to identify c-Fos activity in the bed nucleus of the stria terminalis (BNST), lateral septum (LS), and the paraventricular nucleus of the hypothalamus (PVN), all areas important to maternal behavior that contain CRH producing cell bodies. Changes in c-Fos activity were not observed between HCRT-1 and vehicle groups. Based on
these results, it is possible that the inhibitory effects of HCRT-1 on maternal defense behavior are likely not driven by CRH receptors, but rather these systems work independently to impair maternal defense.

*Keywords:* maternal behavior, corticotropin-releasing hormone, hypocretin
Introduction

During the early postpartum period, peptides influence maternal care in crucial ways. Following parturition, animals experience large changes in hormones and neuropeptides, related to lactating animals’ increase in states of arousal and wakefulness (Levin & Stern, 1975; Stern & Levin, 1976). This is quite possibly due to the increased need to meet energy demands and attend to offspring (Chiang, Johnson, & Nielson, 2002; Leon & Woodside, 1983). Such changes require more time spent awake and alert. Caring for offspring is associated with increased sleep disruptions and time spent awake (Nishihara & Horiuchi, 1998). However, it is possible that peptide-induced states of hyperarousal can lead to overactivation of stress mechanisms, which may have deleterious effects both exogenously (in terms of behavior, such as providing inadequate care of young) as well as endogenously (physiological ailments and health problems, as well as neuropeptide irregularity). For instance, stressors from the environment can have a negative impact on maternal behavior, and altering stress-related peptides enhances this effect (D’Anna, Stevenson, & Gammie, 2008). The primary peptides of interest for the present study are corticotropin-releasing hormone (CRH) and hypocretin (HCRT). CRH is well known for its involvement with the physiological response to stress via the hypothalamic-pituitary-adrenal (HPA) axis, as well as for behavioral responses to stress, promoting anxiety and hypervigilant states within an organism (Britton, Koob, Rivier, & Vale, 1982; Davis, Cedarbaum, Aghajanian, & Gendelman, 1977; Dunn & Berridge, 1990; Dunn, Berridge, Lai, & Yachabach, 1987; File, Johnston, & Baldwin, 1988; Swerdlow, Geyer, Vale, & Koob, 1986). HCRT is well known for its involvement with arousal (de Lecea et al., 1998; Pañeda, Winksky-Sommerer, Boutrel, & de Lecea, 2005). Much evidence suggests that the two peptides work together, which may provide a neuropeptide basis for the argument of a hyperarousal and hypervigilance model.
HYPOCRETIN-1, CRH, AND MATERNAL BEHAVIOR

of stress. Finally, an interaction between the two peptides might be relevant to maternal behavior.

**Stress as Vigilance and Arousal**

Stress peptides tend to promote states of vigilance and increased arousal. Central injection of CRH induces anxiogenic behavior in rodents (Britton et al., 1982; Davis et al., 1977; Dunn & Berridge, 1990; Dunn et al., 1987). Central CRH can also promote arousal through increased wakeful states (Chang & Opp, 2002; Kimura et al., 2010), whereas blocking CRH receptors promotes sleep (Chang & Opp, 2002; Held et al., 2004). To a certain degree, stress is necessary, healthy, and adaptive. A moderate amount of stress can promote adaptive traits, allowing organisms to thrive in their environment (DiPietro, Novak, Costigan, Atella, & Reuising, 2006). In this vein, stress serves as a psychological thermostat for adaptive behaviors. It may often be the case, however, that this thermostat gets pushed to its maximum due to the bombardment of daily stressors. Excess environmental stress may therefore lead to excess expression of stress-related neuropeptides (Henry, Kabbaj, Simon, Le Moal, & Maccari, 1994; Maccari & Morley-Flethcer, 2007). In the process, individuals may experience states of “hypervigilance” as they become exceedingly watchful over their environment or “hyperarousal” as they experience reductions in sleep and increases in wakefulness (Chang & Opp, 2002; Kimura et al., 2010). In the following pages I will attempt to explain how the actions of HCRT and CRH suggest a hypervigilance-hyperarousal model of stress. I shall then explain how this model can influence maternal behavior.

**Hypocretin and Arousal**

The hypocretins (also known as orexins), which include HCRT-1 (orexin A) and HCRT-2 (orexin B), are two structurally similar neuropeptides derived from the 130-amino-acid
precursor peptide preprohypocretin (de Lecea et al., 1998; Pañeda et al., 2005). HCRT neurons are localized in the lateral hypothalamus (LH), periforntical hypothalamus, and dorsomedial hypothalamus, and they send projections throughout the brain (Espana, Berridge, & Gammie, 2004, de Lecea et al., 1998; Hagan et al., 1999; Marcus et al., 2001; Winsky-Sommerer et al., 2004). Two G-protein-coupled HCRT receptors have been identified (HCRT-r1 and HCRT-r2), which have differential affinities to each HCRT subtype. Both HCRT-1 and HCRT-2 bind with similar high affinities to HCRT-r2 (Sakurai et al., 1998). However, for HCRT-r1, only HCRT-1 binds with comparable affinity to HCRT-r2, whereas HCRT-2 binds with the HCRT-r1 receptor at much lower affinity (100- to 1000-fold) (Sakurai et al., 1998).

HCRT receptors are dispersed throughout the brain, and may serve as neuromodulators. The HCRT receptors are differentially expressed in areas related to arousal, anxiety, response to threat monitoring, metabolic processes, thermoregulation, hormonal secretion, sensory and motor processing, memory, reward and reinforcement, motivation, perceptual awareness, and attention (Marcus et al., 2001). In the rat brain, HCRT-r1 shows light-to-moderate expression in areas of the cortex (attention and perceptual awareness), light-to-strong expression in the amygdala (fear and anxiety) and bed nucleus of the stria terminalis (BNST) (anxiety and threat monitoring), and mostly light with sparse areas of strong expression in the thalamus (sensory and motor processing) (Marcus et al., 2001). HCRT-r1 also shows light-to-strong expression in the hippocampus (memory) and septum (reward), moderate-to-strong expression in the hypothalamus (metabolic processes and hormonal secretion), light-to-moderate expression in preoptic nuclei (thermoregulation), and moderate-to-strong expression in many areas of the midbrain (motivation). In the locus coeruleus (LC) (arousal) and other noradrenergic areas such as cell groups A4, A5, and A7, HCRT-r1 shows remarkably strong expression (Marcus et al.,
HCRT-r2 shows sparse areas of light-to-moderate expression in the cortex, amygdala, and BNST, light-to-strong expression in the hippocampus and septum, and slightly more expression in the thalamus and preoptic area than HCRT-r1 (Marcus et al., 2001). Light and moderate expression of HCRT-r2 can be found throughout the midbrain, pons, and medulla as well. Furthermore, unlike HCRT-r1, HCRT-r2 shows mostly moderate and strong expression in many areas of the hypothalamus, especially paraventricular areas and LH, but only light expression in the LC, and no expression in norepinephrine-rich areas such as A4, A5, and A7 (Marcus et al., 2001). Additionally, infusions of HCRT-1 into the lateral ventricles in postpartum mice show increase c-Fos expression in areas relevant to maternal behavior, such as the olfactory bulb, cingulate cortex, lateral septum, suprachiasmatic nucleus, anterior hypothalamus, ventromedial hypothalamus (VMH), paraventricular thalamus, subthalamic nucleus, and LC (D’Anna & Gammie, 2006).

Evidence suggests that HCRT is involved with the maintenance of arousal by keeping animals awake and alert (Pañeda et al., 2005). HCRT-1 increases arousal and locomotor behavior by acting on the LC, an area rich in norepinephrine and known to be involved with arousal (Hagan et al., 1999). Infusions of HCRT-1 into the medial septal area, the medial preoptic area (MPOA), and substantia innominate potentiate wake states in rats (España, Baldo, Kelley, & Berridge, 2001). Narcoleptic canines demonstrate deficient HCRT-r2 receptors (Lin et al., 1999), and REM sleep is significantly increased in preprohypocretin knockout mice (Chemelli et al, 1999). Additionally, both HCRT subtypes have demonstrated primarily excitatory effects on efferent neurons (Samson et al, 2002; Shirasaka et al., 2001), and HCRT neurons themselves tend to be primarily excitatory
Together, these studies suggest an excitatory action of HCRT and HCRT neurons on the central nervous system in areas relevant for maternal behavior.

Corticotropin-Releasing Hormone (CRH) and Behavioral Response to Stress

CRH is a 41-amino-acid polypeptide found in the hypothalamus, and is involved with the behavioral response to stress and anxiety (see below) and the release of hormones in the HPA-axis, such as anterior pituitary hormone adrenocorticotropic (ACTH) (Stenzle-Poore, Duncan, Rittenberg, Bakke, & Heinrichs, 1996). CRH has two receptor subtypes (CRH-r1 and CRH-r2) that are widely distributed throughout the brain (Van Pett et al., 2000). In both rats and mice, CRH-r1 shows expression in areas of the cortex, olfactory regions, hippocampus, amygdala, septum, basal ganglia, thalamus, hypothalamic regions, brain stem and cerebellum. CRH-r2 shows similar expression in the hippocampus, amygdala, hypothalamus, and septal regions, but less expression in the cortex, olfactory regions, basal ganglia and brain stem, and is absent in the thalamus and the cerebellum (Van Pett et al., 2000). Much research shows that laboratory-induced stressors alter CRH receptor expression and increase CRH release in the brain (Giardino, Puglisi-Allegra, & Ceccatelli, 1996; Hauger, Lorang, Irwin, & Aguilera, 1990; Makino et al., 1995; Merlo Pich et al., 1995).

CRH produces anxiogenic and stress-like behavior in animals, implicating its involvement in the behavioral response to stress. Infusion of CRH into the LC increases anxiety-like behavior in rats (Butler, Weiss, Stout, & Nemeroff, 1990). Central administration of CRH into the lateral ventricles dose dependently affects locomotor activity in the center portion of an open-field apparatus, indicative of anxiogenic behavior. In the open field, lower doses CRH increase, whereas higher doses decrease, locomotor behavior in both rats (Sutton, Koob, Le Moal, Rivier, & Vale, 1982), and mice (Lee, Tang, & Chai, 1987). The effect of the lower dose
of CRH increasing time spent moving about the center portion of an open field mimics the effects seen on this test after stress induction by immobilization and footshock stress (Lee, Tsai, & Chai, 1986). The dose-dependent effects of CRH infusions suggest that the peptide shows an inverted-U pattern of influence on behavior, such that low doses promote boldness and higher doses promote anxiety.

Several other behaviors indicative of anxiety are facilitated by CRH administration into the lateral ventricles. Self-grooming is associated with stress or anxiety (Dunn & Berridge, 1990), as grooming increases in response to presentation of a novel environment, and decreases following habituation to such an environment (Colbern, Isaacson, Green, & Gispen, 1978) or administration of anxiolytic drugs (Dunn, Guild, Kramarcy, & Ware, 1981). Accordingly, i.c.v. administration of CRH dose-dependently increases self-grooming behavior in mice and rats (Britton et al., 1982; Dunn et al., 1987). Startle in response to an acoustic stimulus, another stress-related behavior (Davis et al., 1977), is intensified following administration of CRH into the lateral ventricles of rats (Swerdlow et al., 1986). Anxiety can also be measured by the amount of time animals spend in the closed versus open portions of an elevated plus maze. Less time spent in the open portion indicates greater anxiety. CRH significantly decreases time spent in the open arms of an elevated plus maze (File et al., 1988; Spina et al., 2002). Furthermore, α-helical CRH, a nonselective CRH receptor antagonist, decreases withdrawal behavior into a metal cylinder in an expansion of the open-field test, whereas CRH administration can reduce the effect of habituation-induced decreases in withdrawal on this test (Takahashi, Kalin, Vanden Burgt, & Sherman, 1989). The effect of increased defensive withdrawal has been replicated with CRH as well as CRH-related peptide, urocortin (Ucn) (Spina et al., 2002).
CRH can also produce inhibitions of certain behaviors in ways that support its anxiogenic role. In a Geller-Seifter conflict test, an animal receives electric shock for engaging in a previously reinforced behavior, such as lever presses for food (conflict or punishment portion) or the animal freely presses the lever for food without any punishment (no-conflict portion). Rat responses (lever presses) during the punishment portion of this test typically increase after administration of minor tranquilizers (Britton, Morgan, Rivier, Vale, & Koob, 1985; Dunn & Berridge, 1990), an effect not observed during the no-conflict portion of the test. Increased lever pressing during the conflict portion indicates less anxiety, whereas a decrease in lever pressing indicates anxiety provocation. Administration of CRH into the lateral ventricles significantly decreases lever pressing during the conflict portion (Britton, Lee, & Koob, 1988; Spina et al., 2002), an effect that is reversed by benzodiazepines (Britton et al., 1988; Britton et al., 1985). The anxiogenic properties of CRH are further supported by observations of decreased social interactions between rats in an open-field in response central administration of CRH (File et al., 1988), which can also be reversed by administering a benzodiazepine (Dunn & File, 1987).

In light of the aforementioned studies, it becomes clear that CRH is a peptide that not only induces the release of stress-related hormones from the HPA-axis, but can also work in a neurotropic fashion to potentiate stress-related behaviors in rodents. CRH may therefore be thought of as a stress-related peptide both physiologically and behaviorally. The present study focuses on a model that defines stress as a state of hyperarousal and hypervigilance. Whereas vigilance and arousal are observed by increases in wakeful behaviors (Kimura et al., 2010), and may be necessary for certain adaptive behaviors, such as tending to young pups during the early postpartum period, hypervigilance and hyperarousal may manifest negatively from excessive exposure to stress, reducing such adaptive behaviors. Administration of either CRH or HCRT
can produce these effects (Britton et al., 1988; Ida, Nakahara, Katayama, Murakami, &
Nakazato, 1999; Spina et al., 2002; Suzuki, Beuckmann, Shikata, Ogura, & Sawai, 2005).
Therefore, a discussion of the possible interaction between the stress-related peptide CRH and
arousal-related peptide HCRT is warranted.

**Interactions between HCRT & CRH**

Hypocretin and CRH have mutually excitatory actions on one another’s nuclei in the
hypothalamus. HCRT-1 may act directly on CRH neurons in the PVN via cell bodies or
dendrites, which contain HCRT-r2 receptor subtype (Trivedi, Yu, MacNeil, Van der Ploeg, &
Guan, 1998). Electrophysiological studies have revealed such excitatory roles of hypothalamic
HCRT and CRH neurons on one another’s neurons. For example, HCRT peptide has a primarily
excitatory effect on CRH neurons in the PVN (Samson, Taylor, Follwell, & Ferguson, 2002),
and CRH excites HCRT cells in the LH (Winsky-Sommerer et al., 2004). Whole cell recordings
in the PVN of rats show that approximately two-thirds of the cells depolarize as a result of
HCRT-1 bath applications, occurring within one minute of the application, and lasting anywhere
from three to ten minutes (Samson et al., 2002). In that study, depolarization was indeed induced
by HCRT-1, and not by activity of adjacent neurons, as individual cells were isolated by
temporarily blocking peripheral cells using tetrodotoxin (TTX). The same ratio of these isolated
cells depolarized as a result of HCRT-1 bath application. In a separate study, eight out of 32 *ex
vivo* HCRT neurons taken from the hypothalamus depolarized in response to bath application of
CRH (Winsky-Sommerer et al., 2004). Depolarization of the HCRT neurons last as long as CRH
is present, and ceases when slices are washed clean. Additionally, applying astressin, a selective
CRH-r1 antagonist, to the bath significantly inhibits neural firing by HCRT neurons.

Anatomical connections between HCRT and CRH have also been established. In rats,
both HCRT-1 and HCRT-2 neurons from the perifornical region of the LH show fiber projections to the PVN (Date et al., 1999). Immunolabeling procedures confirm that CRH axons are abundant in the LH, where many HCRT-immunoreactive (ir) neurons are observed (Winsky-Sommerer et al., 2004). HCRT fibers can be seen in the PVN and central nucleus of the amygdala (CeA), where CRH neurons are present. Additionally, electron microscopy analysis of CRH-ir bouton-like structures (bulges at axon terminal) suggests that CRH neurons synapse onto HCRT-ir cell bodies, and furthermore, the synapses are primarily excitatory (Winsky-Sommerer et al., 2004).

Double-label immunohistochemistry studies have investigated HCRT’s effect on CRH neuronal activity. Sakamoto, Yamada, and Ueta (2004) administered HCRT-1 centrally in rats, and double-labeled for c-Fos and CRH in the parvocellular region of the PVN (pPVN), as well as in the CeA. Double staining of c-Fos and CRH would indicate recently activated cells in these regions. HCRT-1-injected rats demonstrated significantly more double-immunoreactivity of CRH and c-Fos in the pPVN and CeA. For HCRT-1 injected animals, 95.9% of CRH-ir cells in pPVN showed c-Fos-ir, whereas only 14% of pPVN CRH cells were double-labeled in saline-injected controls. In the CeA, 44.5% of the CRH-labeled cells demonstrated c-Fos-ir for HCRT-1 injected rats, whereas control animals show double labeling in 5.6% of cells. One notable observation regarding this study is the particularly strong effect sizes for the mean differences in percent of c-Fos positive CRH neurons ($d_{pPVN} = 21.06$ and $d_{CeA} = 5.89$). The authors provide strong evidence in support of the hypothesis that HCRT acts on CRH neurons in the PVN, although it should be noted that while c-Fos activity indicates neuronal activity, it does not indicate whether or not the neurons have excitatory or inhibitory effects. However, aforementioned studies show that HCRT-1 depolarizes neurons in the PVN, indicating an
excitatory input by the neuropeptide (Samson et al., 2002). Therefore, it is likely that HCRT acts on CRH neurons in the PVN via excitatory inputs. The activating effects of HCRT-1 on CRH neurons in CeA may be excitatory as well, which could also implicate HCRT-1 in not only the physiological responses to stress, but also emotional behavioral responses, such as fear and avoidance (Sakamoto et al., 2004).

CRH is at the head of the HPA axis, signaling from the hypothalamus to the anterior pituitary to release ACTH, which then signals cortisol or corticosterone release from the adrenal glands. Several studies have looked at the effects of HCRT administration on HPA axis activity. In rats, i.c.v. administration of HCRT-1 increases plasma levels of the adrenal glucocorticoid corticosterone (Ida et al., 2000b). Both HCRT-1 and HCRT-2 influence adrenal activity when administered to the lateral ventricles, increasing corticosterone levels detected in trunk blood plasma of male rats. This effect is abolished by pre-treatment with CRH antagonist α-helical CRH (Jaszberenyi, Bujdoso, Pataki, & Telegdy, 2000), a result that was replicated by Samson et al. (2002). HCRT-1 has also been shown to affect the release of the anterior pituitary hormone ACTH. Central administration of HCRT-1 dose-dependently raises blood plasma levels of ACTH in rats (Samson et al., 2002). Interestingly, in a prior study, Samson & Taylor (2001) demonstrated ACTH secretion is reduced by both HCRT-1 and HCRT-2 bath applications, although this was done on isolated in vitro anterior pituitary cells. It may be that hypocretins behave differently in the anterior pituitary itself, and may not represent HCRT’s action on the HPA axis in vivo. Perhaps HCRT increases ACTH levels indirectly by stimulating CRH release. In addition, expression of HCRT in the brain can be manipulated by glucocorticoids released from the adrenal glands, as demonstrated in adrenalectomized (ADX) rats (Stricker-Krongrad & Beck, 2002). In ADX rats, areas of the hypothalamus, including LH, show significantly less
preprohypocretin mRNA expression than sham surgery animals. Additionally, preprohypocretin mRNA expression in ADX can be reinstated to levels comparable to controls by administering a synthetic glucocorticoid dexamethasone. It appears that the HPA axis as far down as the adrenal gland has an effect on HCRT activity in the hypothalamus.

Few studies have investigated the interaction between HCRT and CRH in terms of behavior. In rats, Ida et al. (2000b) examined the effect of a CRH antagonist (α-helical CRH) on several behaviors induced by HCRT-1 administration. In that study, HCRT-1 increased anxiety-related behaviors such as face washing, self-grooming, and locomotor behavior. The CRH antagonist reduced all of these behaviors when pre-administered with HCRT, suggesting that HCRT-induced behavior may be moderated by CRH activity. There also appears to be an interaction between HCRT-1 and CRH in terms of feeding behaviors. CRH antagonists produce increased feeding in fasted rats only when paired with HCRT-1, neither of which influence feeding on its own (Ida et al., 2000a). These two studies provide evidence of the two peptides working synergistically to influence behavior, which may manifest in other forms of behavior, although this is yet to be determined.

Stress-exposure studies offer additional information regarding a possible connection between HCRT and CRH via exposure to environmental stressors. As CRH activity is associated with exposure to such stressors (Feng, Vurbic, Wu, & Strohl, 2007; Hayley, Staines, Merali, & Anisman, 2001; Koob & Heinrichs, 1999; Ladd, Owens, & Nemeroff, 1996; Plotsky et al., 2005), stressors may impact HCRT. Rats exposed to immobilization stress show significant increases in preprohypocretin mRNA expression in the LH compared to controls or rats that received cold stress (Ida et al., 2000a). Cold exposure and restraint stress both increase c-Fos-ir in HCRT-1 neurons in the LH (Sakamoto et al., 2004). Additionally, postnatal maternal
deprivation has been shown to increase HCRT-1-ir in the hypothalamus, which is also accompanied by hyperarousal-like alterations of wake and sleep states in rats (Feng et al., 2007). Interestingly, both HCRT and CRH both show increases in expression in response to maternal HCRT-related arousal is altered in specific ways depending on the type of stress exposure. Steiner et al. (2012) demonstrated that almorexant, a dual hypocretin antagonist that blocks HCRT-r1 and HCRT-r2, significantly decreased time actively awake, and reduced locomotor behaviors in novelty and socially stressed rats. Additionally, almorexant increased time spent in non-REM sleep in a novelty stress condition (placed in an empty, unfamiliar cage without bedding), whereas the drug increased quiet wake time in the social stress condition. Social stress, however, had no effect on REM or non-REM sleep times, whereas novelty stress had no effect on REM or quiet wake times for animals treated with almorexant. For novelty or social stress conditions, almorexant also shortened the latency to the first persistent non-REM sleep episode, but had no effect on the latency to the onset of the first REM sleep episode. Furthermore, almorexant did not affect latencies to the onset of the first persistent REM or non-REM sleep episode, nor time spent in any vigilance states or sleep stages, for rats in the restraint stress condition. These observations suggest that different forms of stress might differentially affect the ability of HCRT to modulate sleep and wake states, and may be indicative of the differential effectiveness of each type of stressor at producing states of arousal in the animal.

Differences in HCRT expression are also observed on the neural level in response to different stressors. Ida et al. (2000a) showed that immobilization, but not cold stress, significantly altered hypocretin expression in the hypothalamus. Winsky-Sommerer et al. (2004) compared CRH-r1 knockout mice to wild-type mice in terms of hypocretin and c-Fos immunoreactivity induced by different stressors. The authors observed significantly more
HCRT-ir showing c-Fos-ir in the perifornical region of the LH for wild-type mice compared to knockouts after both restraint stress and footshock. Stressors did not appear to significantly alter HCRT expression for CRH-r1 knockout mice when compared to animals that received no stress (control wild-type and control knockouts). The evidence suggests that CRH-r1 may be a necessary component for HCRT's ability to respond to stress. Furthermore, different environmental stressors might mediate HCRT expression in ways unique to specific type of stressor.

Rodents also display anxiety-like behaviors after receiving HCRT in ways similar to CRH administration. In rats, i.c.v. HCRT-1 increases grooming and burrowing behavior, whereas HCRT-2 increases burrowing and searching behavior (Ida et al., 1999), and both hypocretins dose dependently induce freezing and grooming behavior when injected into the paraventricular portion of the thalamus (PVT), an area that innervates with the amygdala (Li et al., 2010a). In mice, HCRT-1 increases time spent in the dark portions of a light-dark test (animals avoid the light area) and increases time spent in the closed arms of an elevated plus-maze apparatus (Suzuki et al., 2005). HCRT-1 and HCRT-2 infusions into the PVT decrease time spent in the open arms of an elevated plus maze in rats (Li et al., 2010b). Both hypocretins also dose-dependently reduce exploration of the center portion of an open field and novel object (Li et al., 2010a). Collectively, these studies suggest that the hypocretins facilitate a hypervigilance state as observed by anxiety-like behaviors. The similarity between HCRT and CRH based on their ability to produce anxiety-like behaviors provides further support for a behavior-relevant interaction between these two peptides.

**HCRT, CRH, and Maternal Behavior**
The postpartum period for mice and rats consists of behaviors largely focused on taking care of pups. Dams will spend a great deal of their time on the nest engaging in pup-directed maternal care such as hovering over pups, nursing, licking and grooming pups, retrieving pups back to the nest, and nest building (Rosenblatt, 1967). Maternal defense is another maternal behavior that is characterized as aggression toward unfamiliar conspecifics, which protects young against infanticide (Wolf, 1985; Agrell, Wolff, & Ylonen, 1998). Studies have found similar effects of either HCRT or CRH on maternal defense in postpartum dams. Maternal defense is measured by latency to attack (shorter latencies indicate greater aggression), number of attacks, and total time spent being aggressive. In mice, i.c.v. injections of either HCRT-1 or CRH dose dependently impair maternal defense, as dams show reductions in time in aggression and number of attacks, although only CRH, and not HCRT, increases the latency to attack (D’Anna & Gammie, 2006; Gammie, Negron, Newman, & Rhodes, 2004). Urocortin 1 (Ucn1) and urocortin 3 (Ucn3) are sister peptides to CRH, binding to its receptors (Hsu & Hsueh, 2001; Lewis et al., 2001; Vaughan et al., 1995). Both of the urocortins impair maternal defense (D’Anna, Stevenson, & Gammie, 2005). Lactating dams that receive centrally administered Ucn1 show significant impairment of maternal defense on all three measures, whereas higher doses of Ucn3 are needed for animals to show impairment of maternal defense, suggesting that Ucn1 is more potent at affecting defense behavior in mice (D’Anna et al., 2005).

Collectively, these studies showing impairment of defense behavior suggest that a down-regulation in the peptides is required for normal levels of maternal defense to take place (D’Anna et al., 2008), although an up-regulation of HCRT may be present in lactating animals (see below). Relatedly, CRH antagonism has no effect on basal levels of maternal defense (Gammie et al., 2004). This might be expected because CRH and other HPA axis related-peptides are
already down regulated in lactating rodents (da Costa, Kampa, Windle, Ingram, & Lightman, 1997; da Costa, Wood, Ingram, & Lightman, 1996). It should be noted, however, that a certain amount of CRH is necessary for normal levels of maternal defense, as CRH-r2 knockout mice exposed to a mild stressor display longer latencies to attack, spend less time in aggression, and show a trend for decreased number of attacks (D’Anna, Stevenson, & Gammie, 2008). A particular amount of CRH is necessary for optimal maternal defense, whereas excessive elevations reduce the behavior, a pattern similar to the dose-dependent effect of HCRT on maternal behavior (D’Anna & Gammie, 2006).

Virgins and lactating animals differ in how they respond to pups, which may be mediated by glucocorticoids (Rees, Panesar, Steiner, & Fleming, 2004; Rees, Panesar, Steiner, & Fleming, 2006). Both HCRT and CRH expression change during lactation. CRH appears to be down regulated during lactation (da Costa et al., 1997; da Costa et al., 1996), whereas HCRT production is increased (España et al., 2004). Although virgin and lactating mice have similar cell counts of HCRT-synthesizing neurons in the LH, lactating dams show marked increases in c-Fos-ir in LH neurons in general, as well as an increased percentage of c-Fos-ir in HCRT-1 neurons within the LH (España et al., 2004). In the same study, the MPOA, an area important to maternal behavior that is innervated by HCRT, also showed increased c-Fos-ir for lactating mice. Interestingly, the LC does not show differential expression between virgins and lactating mice. However, perfusions were performed during light hours, when animals tend to be less active. Although dams spend more time with their litters during the day, dams are typically more active at night, and spend significantly more time eating nocturnally than diurnally (Levin & Stern, 1975; Stern & Levin, 1976). This suggests a nocturnal increase in wake state is necessary to consume and provide an appropriate amount of nutrients for mother’s offspring. In fact, HCRT-1
neurons in the LC show the greatest c-Fos expression nocturnally in rats (España, Valentino, & Berridge, 2003), an effect that could possibly be potentiated in lactating dams, although this is to be determined by future studies. As a potential neuromodulator, HCRT may be upregulated to promote certain behaviors related to maternal care, which may be contingent upon the time of day when the behaviors occur. Furthermore, although CRH and HCRT show opposite trends of expression during lactation, this seems to make sense in terms of maintaining optimal levels of behaviors with which the peptides are associated. However, when excess levels of HCRT are present, disruptions of CRH homeostasis may occur, facilitating CRH action beyond the basal levels, thereby reducing maternal behavior.

The inverted-U pattern of HCRT’s influence on maternal behavior has been demonstrated in lactating dams (D’Anna & Gammie, 2006), although the role of CRH as an intermediary in this process is still to be determined. A high dose of HCRT-1 administration (0.3μg) diminishes maternal defense and decreases nursing behaviors. Dams take longer to initiate nursing, spend less time engaged in total nursing behavior, and spend more time off of the nest. Again, decreases in defensive behaviors are demonstrated by fewer attacks and less time spent being aggressive for the 0.3μg HCRT-1 dose. Conversely, moderate levels of HCRT-1 administration (0.1μg) do not alter maternal defense behavior, but do facilitate pup-directed maternal behavior. The 0.1μg dose leads to larger number of nursing bouts, as well as more time spent in high-arched back nursing. Indeed, the effects of HCRT-1 on maternal behavior operate in an inverted-U fashion, facilitating maternal behavior with moderate increases, but diminishing them at higher levels of administration. Perhaps a moderate boost in HCRT-related arousal might facilitate maternal behavior, whereas higher levels of HCRT-related arousal lead to a reduction in maternal behavior, possibly by activating CRH. However, the extent to which HCRT-1 interacts
with CRH in the context of maternal behavior is unexplored, and is the primary purpose of this study.

**Justification for Current Study**

It is important that the effects of an interaction between CRH and HCRT is understood, as hyperactivity of these peptides brought out through stressful circumstances may lead to deleterious effects on offspring. Exposing pregnant rats to stressors can have long lasting deleterious effects on future maternal behavior of their offspring, can elevate HPA axis correlates, and can increase anxiety in response to acute stressors, thus priming the offspring to be more reactive to stress and prone to anxiety (Bosch et al., 2007). This is quite possibly due to prenatal exposure to CRH and/or other stress-related peptides in utero. Indeed, in pregnant women, elevated cortisol, an indirect indicator of increased CRH activity, is associated with larger amygdala volumes and more affective problems in offspring (Buss et al., 2012). Additionally, maternal mood states might affect offspring as well, as cortisol irregularity in children is associated with maternal depression in women living in low socioeconomic conditions (Fernald, Burke, & Gunnar, 2008). As mentioned before, maternal deprivation in rats can lead to elevations in CRH and HCRT-1 in expression, which is accompanied by hyperarousal states (Feng et al., 2007). Therefore, it is necessary to understand how these peptides work together to affect maternal behavior.

Excess levels of HCRT-1 administration lead to reductions in maternal behavior and maternal defense, yet it is unknown whether or not the HCRT-1 peptide is working in concert with the CRH system to produce these effects. What is clear is that excess levels of these peptides reduce maternal behavior. In the present study I explored the possibility of CRH working with HCRT-1 in altering maternal behavior. I pretreated primiparous dams with a CRH
global antagonist for receptors 1 and 2 (α-helical CRH), and then administered HCRT-1 at a level shown to decrease maternal defense and pup-directed maternal behavior. I hypothesized that blocking CRH would lead to an increase in maternal defense and pup-directed maternal behavior. Additionally, using immunohistochemistry for c-Fos, I examined the influence of the same dose of HCRT-1 on activity of neurons in brain areas where CRH receptors are present. I predicted that animals receiving the HCRT-1 dosage would show marked increase in c-Fos activity in these regions as well as in areas important to maternal behavior compared to mice receiving vehicle injections where CRH receptors are also present. Answering these inquiries help to elucidate the interwoven nature of HCRT and CRH in relation to maternal behavior.

**Methods**

**Subjects**

Forty female mice (*Mus musculus*) (hsd:ICR) were paired at 42-56 days of age with sexually experienced males of the same strain, and 27 females gave birth to pups. All mice were housed on a 14:10 h light/dark cycle (lights on at 0630 h PST), and access to Breeder Chow (Harlan) and tap water were made available *ad libitum*. On day 10 after pairing, males were removed from the experiment, and females were placed in a new polypropylene cage with precut nesting material; cages remained unchanged throughout the rest of the experiments. Once born, litter size was culled to 9-12 pups in order to reduce effects of litter size and assure that pups had adequate nursing access. Sexually naïve males of the same strain (approximately 42-56 days postpartum) were used as intruder males for the maternal defense tests. Intruders were housed two to four mice/cage with littersmates. Testing was done between 09:00 and 15:00h in a separate testing room outside of the vivarium, and surgeries were performed in a separate surgery suite located in the vivarium outside of the mouse colony facility. All procedures followed the
guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Care and Use Committee of California State University, San Marcos.

**Surgeries**

Surgical procedures for this study were performed as previously established (D’Anna & Gammie, 2006). On postpartum day 1, primiparous mice were anesthetized with isoflurane, and cannulas for intracerebroventricular (i.c.v.) injections were implanted into the lateral ventricles. Pups were kept warm with a lamp. While under isoflurane anesthesia, hair was removed from the top of the head with an electric trimmer, and cleaned with alcohol and Betadine (Purdue Frederick, Stamford, CT, USA). Using a stereotaxic apparatus (David Kopf Instruments), an incision along the midline over the skull was made and periosteum removed. A hole was drilled (1mm) at – 0.6 mm (posterior) to and 1.6 mm (lateral) to Bregma. A 26 gauge stainless-steel indwelling cannula was implanted (Plastics One, Roanoke, VA, USA) to –2.5 mm below the skull into the lateral ventricle. Two small screws were set into the skull by drilling and implanting the screws lateral and anterior and posterior to the cannula site to stabilize and secure dental cement to the skull. On days of injections, a dummy cannula was inserted to aid with injections, which was made with a 33-gauge stainless-steel injector extending 1 mm beyond the guide and attached to PE-50 tubing (Becton Dickenson, Sparks, MD, USA) fitted to a Hamilton syringe. After surgery, animals were given ketoprofen (3mg/kg, MP Biomedicals, LLC, Solon, Ohio), a non-steroidal anti-inflammatory used to relieve pain, swelling, tenderness, and stiffness, and were put back in the home cage with their pups. All dams were given two days to recover before given i.c.v. injections. Cannula placement was verified using injections of Chicago sky blue dye before brain extractions (refer to immunohistochemistry methods).
Intracerebroventricular injections (i.c.v.) of HCRT-1 and CRH antagonist

Two days after recovery from surgery, i.c.v injections began and took place for three consecutive days. The CRH antagonist used in this study was α-helical CRH, which is a global CRH-receptor antagonist and thus blocks both 1 and 2 receptor subtypes of CRH. On the first day of injections, animals were randomly selected to undergo one of three testing schedules: (1) α-helical CRH at high dose (5µg) + HCRT-1 (0.3µg); (2) α-helical CRH at low dose (2.5µg) + HCRT-1 (0.3µg); or (3) saline + HCRT-1 (0.3µg) (D’Anna & Gammie, 2006; Stenzel-Poore et al., 1996). After verification of cannula placement and aggression levels (see Results), the final number of animals included in this injection paradigm was $n = 7$ for maternal defense and $n = 11$ for maternal behavior. To administer injections, animals were anesthetized with isoflurane and injected with either saline or global CRH antagonist at a low dose or high dose. After 15 minutes, all animals were anesthetized again with isoflurane, and given HCRT-1 (0.3µg). Over the course of three days, each dam was exposed to all three testing schedules counterbalanced, employing a repeated-measures design. Between injections, animals were placed back in the home cage with their pups. Injections were made using a 1μl volume over a 60 second time span while under light anesthesia. Infusions were verified by following the movement of an air bubble in the tubing with the injector remaining in place for 60 seconds following each injection.

Maternal defense and behavior tests

Both maternal defense and behavior tests were recorded with digital video cameras (Canon Vixia HF R32 HD CMOS). Maternal defense and behavior testing occurred on postpartum days four, five, and six, 20 minutes following final i.c.v. injection. For the maternal defense test, dams remained in their home cage, all pups were removed, and an intruder male was placed in the home cage for five minutes. Behaviors were recorded on video and scored later
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(while blind to condition) for maternal aggression, including latency to first attack, number of attacks, and total duration of attacks. An attack consists of lunging toward male, biting male, or clawing at face or body. Self-grooming and tail rattling were used as ancillary behavioral measures of stress-like behavior and threat-like behavior respectively. Only mothers that displayed at least 5 seconds of aggression on at least one day of testing were included in the aggression analysis as to prevent floor effects in aggression ($n = 13$).

Upon completion of the maternal aggression test, males were removed, pups were scattered around the home cage, and the maternal behavior test ensued for 30 minutes. Latency to retrieve first, second, third, fourth, and all pups, latency to nurse, number of nursing bouts (a nursing bout lasts until all pups are off the nipples), duration of each nursing bout, total time nursing, time spent in arched back nursing position (wherein dams display an arched back posture and legs are splayed over the pups), or supine nursing (dam lays on their side while nursing), total duration of nest building activity, total duration of licking and grooming of pups by the dam (LG) while either on nest, nursing, or not nursing, latency for the dam to be on nest, total time spent on the nest, time off nest, and total time of self-grooming were examined.

**Immunohistochemistry for c-Fos in CRH receptor brain regions**

Two days after the last behavioral test (PND 8) the mothers were randomly assigned to receive either vehicle or HCRT-1 injection ($0.3\mu g$) to examine how HCRT-1 administration to the lateral ventricles influences c-Fos expression in CRH receptor rich areas of the brain in lactating dams. No behavioral tests were performed. Before perfusions, animals were injected with either vehicle or HCRT-1 ($0.3\mu g$). Cannula placement was verified after testing was completed, and just before fixation, by injecting a $1\mu l$ volume of 0.01% Chicago sky blue (Sigma, St Louis, MO, USA) in saline into the brain. Blue dye present in the lateral ventricles as
seen while cutting the brain on the cryostat or after sections were mounted indicates proper cannula placement. Only correct cannula placements into the lateral ventricles were used for the analyses (see Figure 1). Animals were anesthetized via isoflurane, and lethally injected with sodium pentobarbital (100-200 mg/kg) before perfused transcardially with 4% paraformaldehyde approximately 105 minutes after injection of drug. Brains were placed in 4% paraformaldehyde in a scintillation vial for 24 hours at 4°C. Following this, brains were put in 30% sucrose solution, changed twice over two days before being removed from the refrigerator and placed in a -80°C freezer until used for cutting. Forty-micron sections were cut on a cryostat and stored in cryoprotectant. After this, sections underwent staining for c-Fos using immunohistochemistry. Sections were pretreated with .5 % H2O2, treated with blocking solution (3% normal goat serum and .25% TritonX in PBS), and incubated with anti-c-fos antibody (Santa Cruz Biotechnology, catalog # SC-253, 1:5,000) for two days at 4°C. Sections were then treated with biotinylated goat/donkey anti-rabbit antibody (Vector Laboratories, Burlingame, CA, catalog # BA-1000, 1:200) for 1 hour and with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as dictated by manufacturer protocols. Antibody binding was visualized using 3-3’ – diaminobenzidine (DAB)/Nickel solution, rendering a black/purple precipitate. Following the immunostaining procedures, sections were mounted onto glass slides, dehydrated in a series of alcohol and xylenes, and coverslipped.

Analysis of c-Fos Immunoreactivity

c-Fos positive cells in the lateral septum (LS), bed nucleus of the stria terminalis (BNST), and the paraventricular nucleus of the hypothalamus (PVN) were counted using a microscope with digital image capability (Zeiss with AxioCam MRc5 and Zen Lite 2011 software). Specific sections were used for cell-count analysis in these areas, which include one section of the PVN
and BNST, and three sections of the LS. The sections correspond to the mouse Allen Brain Atlas (http://mouse.brain-map.org) sections as follows: for the LS we analyzed sections 87 – 89 in the atlas, for BNST section 80, and PVN section 68 of the mouse brain. Snapshots of these areas were taken at 20X optical zoom for PVN and BNST and 10X optical zoom for LS, and were saved. Using Zen Lite 2011 software, cells that showed dark enough staining were selected and used to establish a threshold for the quantification of c-Fos-positive cells. These thresholds (Red: 442, Green: 656, Blue: 632) were used throughout to give a total count of the cells. The comparisons made were saline ($n = 8$) to HCRT-1 ($0.3\mu g; n = 4$) injected animals on number of c-Fos-positive cell counts.

**Statistical Analyses**

Behavioral data were analyzed in SPSS (SPSS Inc., Chicago, IL, USA), testing for main effects of HCRT-1 and CRH antagonist on maternal defense including latency to first attack, number of attacks, and total time in aggression, as well as aforementioned pup-directed maternal behavior. Data were not normally distributed; therefore, Friedman’s rank tests for dependent samples were used to test the main effects of CRH antagonist on maternal aggression and maternal behavior, and Mann-Whitney U tests were used to compare vehicle injected animals to HCRT-1 ($0.3\mu g$) injected animals on total c-Fos-labeled neurons.

**Power Analysis.** A minimum of thirty female mice needed to be paired with males in order for this study to achieve optimal power. We assumed *a priori* that parametric tests would be employed, and based the power analysis on these statistical tests. Within-subjects repeated measures analysis ANOVA was the assumed statistical test to test the effect of global CRH antagonist ($\alpha$-helical CRH) pretreatment on measures of maternal behavior and maternal defense in dams injected with HCRT-1 into the lateral ventricles. There is one independent variable with
three levels (αhCRH high-dose, αhCRH low-dose, and vehicle). For this model, G*Power 3.1 found that a minimal sample size of 6 animals would achieve 80% power at a large effect size of 0.8 when using a repeated measures $F$ with a significance level (alpha) of 0.05. Effect size estimate with respect to $F$ was based on calculations of previous similar studies with a range of $d$ from 0.86 to 1.83 (Ida et al., 2000a; Ida et al., 2000b; Samson et al., 2002; Asakawa et al., 2001; Bakshi et al, 2002). This study met the power criteria for both maternal defense ($n = 7$) and maternal behavior ($n = 11$). An independent samples $t$-test was the assumed test in the power analysis in order to test the effect of HCRT-1 on increasing the number of c-Fos positive cells in aforementioned brain areas. A particularly large estimated effect size ($d = 1.5$) was anticipated for this model based previous work with c-Fos labeling procedures, with $d$ ranging from 0.96 to 21.1; the majority of effect sizes were around 1.5 (Sakamoto et al., 2004; Porter & Hayword, 2011; Takahashi et al., 2011; Fekete et al., 2009). G*Power 3.1 calculated a minimal sample size of 14 animals to achieve 80% power at an effect size of 1.5 using independent samples $t$ at an alpha 0.05. For the neuronal comparisons the study fell short of adequate power based on the expected effect size, as after attrition only 12 animals were available for c-Fos comparisons.

**Results**

**Sample Sizes**

We paired a total of 40 females with breeder males, half of which were paired a week before the rest to prevent a potential over-inundation of surgeries. As previously noted, 27 females gave birth out of the 40, two of which were excluded from the study for cannula placement problems (cannulas came out day after the surgery) and one that did not have a large enough litter size. This yielded 24 animals available for the study. Further attrition of animals in each aspect of the study is discussed in the next few sections. Briefly, the total number of
animals was maternal defense test \( n = 7 \); maternal behavior test \( n = 11 \); and immunohistochemistry \( n = 12 \).

**Effects of CRH Antagonist following HCRT-1 (.3µg) injection on Maternal Defense**

Maternal defense tests took place on PND 4, 5 and 6, which were followed by maternal behavior tests immediately afterward. As mentioned above, dams had to display at least five seconds of aggression toward male conspecific on at least one day of testing in order to be considered for the maternal aggression analysis between drug treatment conditions. Out of the 24 lactating dams, 10 did not display at least 5 seconds of aggression and were therefore excluded from the defense portion of the analysis, yielding \( n = 14 \). Furthermore, only those animals with cannula placements into the lateral ventricles could be used. Seven did not have proper cannula placement, yielding a final sample size of \( n = 7 \) to be analyzed by repeated-measures inferential statistics, which met the criteria for adequate power in this portion of the study (see Power Analysis).

Aggression was measured by the latency to first attack intruder male, duration of each attack, and total frequency of attacks; bouts of self-grooming and tail rattling were also documented. Data distributions for all of these measures were not normal. Therefore, Friedman’s rank test for related samples was used in order to investigate the effect of the \( \alpha h \text{CRH} \) at 2.5 µg and 5.0 µg, and vehicle across testing days for animals receiving HCRT-1 (0.3µg). According to the Friedman’s rank test, the CRH antagonist had no effect on latency to first attack (\( \chi^2 = 1.775, df = 2, p = 0.416 \); see Figure 2), duration of attacks (\( X^2 = 3.569, df = 2, p = .168 \); see Figure 3), or duration of self-grooming (\( \chi^2 = .160, df = 2, p = .923 \); see Figure 4). However, there was a significant main effect of CRH antagonist on frequency of attacks (\( \chi^2 = 6.240, df = 2, p = 0.044 \); see Figure 5) and tail rattling (\( \chi^2 = 6.950, df = 2, p = 0.031 \); see Figure 6). Post-hoc comparisons
using Wilcoxon’s Signed-Ranks tests revealed that at 5.0µg αhCRH frequency of attacks was significantly reduced compared to the 2.5µg dose of αhCRH (z = -2.243, p = .025), whereas attack frequency was not significantly lowered at the 5.0µg dose of αhCRH antagonist compared to vehicle-injected animals (z = -.1649, p > .05; see Figure 5). For tail rattling, the same pattern of results was seen, such that 5.0µg of αhCRH antagonist significantly decreased tail rattling compared to 2.5µg of αhCRH antagonist, whereas no such difference was observed between the high dose of CRH antagonist and vehicle (z = -.905, p > .05; see Figure 6).

**Effects of CRH Antagonist following HCRT-1 (0.3µg) injection on Pup-Directed Behaviors**

Out of the 24 dams, 11 were available for maternal behavior analysis after accounting for attrition, which met the criteria for adequate power in the analysis. Data were not normally distributed so Friedman’s rank test was used to test for differences among 2.5µg αhCRH antagonist, 5.0µg αhCRH antagonist, and vehicle treatments for animals treated with HCRT-1 (0.3µg). According to the analysis, CRH antagonist did not significantly affect any of the behavioral measures during the maternal behavior test. However, there was a trend for a main effect of CRH antagonist treatment to decrease total time spent nursing (2.5µg αhCRH, M = 322.36, SD = 414.34; 5.0µg αhCRH, M = 348.64, SD = 424.68; Vehicle, M = 678.82, SD = 371.28, χ² = 5.59, p = .061) and increased latency to begin nursing (2.5µg αhCRH, M = 1281.55, SD = 533.00; 5.0µg αhCRH, M = 1180.73, SD = 628.71; Vehicle, M = 644.91, SD = 570.14, χ² = 5.59, p = .061) (see Table 1 for summary of Friedman’s rank test results).

**Effects of HCRT-1 (0.3µg) on c-Fos expression in CRH-rich areas**

On PND 8, we performed a final injection of HCRT-1 at 0.3µg or injected with saline, and extracted brains to stain for c-Fos expression. Of the 24 animals, 12 had successful lateral ventricle injections [0.3µg HCRT-1 (n =4); saline (n = 8)], which fell below the criteria for
animals needed in this portion of the study \( (n = 14) \), and thus these results did not have adequate power. The cell counts were not distributed normally and, therefore, Mann-Whitney U tests were performed to compare counts between HCRT-1 and saline for the PVN \( (z = -1.021, p = .307) \), BNST \( (z = -1.108, p = .268) \), and three areas of the lateral septum (LS1, \( z = -.340, p = .734 \); LS2, \( z = .000, p = 1.000 \); and LS3, \( z = -1.189, p = .234 \)). None of these areas showed significant alterations in c-Fos expression (see Figures 7 - 11).

**Discussion**

The aim of the present study was to test the hypothesis that high circulating HCRT-1 would decrease maternal behavior and maternal defense in postpartum dams indirectly by activating CRH receptors. A global antagonist to CRH receptors was administered in an attempt to rescue these behaviors. A rescuing effect was not observed for pup-directed maternal behavior or maternal defense; indeed, blocking CRH receptors may further impair maternal defense: while there was no effect on total time attacking or duration of attacks, the highest dose of CRH antagonist decreased the number of attacks on a male intruder relative to the low dose of the CRH antagonist. The same pattern of results was observed in tail rattling, an accompanying measure of threat-like behavior during the maternal defense test. Additionally, blocking CRH receptors before administration of HCRT-1 \( (0.3\mu g) \) had no significant effect on any maternal behavior tests, although there was a strong trend for the antagonist to decrease nursing and increase latency to begin nursing. Furthermore, the dose of HCRT-1 administered before all these tests \( (0.3\mu g) \) did not significantly increase c-Fos activity in the BNST, lateral septum, or the PVN, where CRH-producing cells are abundant. Collectively, the results suggest that HCRT-1 \( (0.3\mu g) \) does not exert its inhibitory effects on pup-directed maternal behavior or maternal defense indirectly via activity of CRH in the brains of postpartum mice. Rather, what I
observed here was a reduction in the maternal profile following reduced activity of CRH in the presence of high circulating HCRT-1.

It has been demonstrated that HCRT-1 dose-dependently alters maternal defense and pup-directed maternal behavior (D’Anna & Gammie, 2006). At moderate increases of HCRT-1 (0.06 – 0.1µg), facilitating effects are observed on pup-directed maternal behaviors, including increases in nursing and licking and grooming of pups. At a higher dose (0.3µg), HCRT-1 impairs both maternal defense and pup-directed behaviors, as demonstrated by decreased number of attacks on male intruder, decreased time spent aggressive toward male intruder, increased latency to begin nursing pups, decreased time spent nursing, especially low arch-back nursing, and increased time spent off the nest away from pups (D’Anna & Gammie, 2006). The present study examined whether the impairing effects of HCRT-1 at the higher dose occurs via activation of CRH cells. It is intriguing that we observed a reduction of attack frequency between doses of the CRH antagonist. Previous work with a CRH antagonist demonstrated no effect on maternal defense behavior (Gammie et al., 2004). Perhaps HCRT-1 is having direct effects on HCRT receptors in areas important to maternal behavior and maternal defense, and that reduced CRH receptor activity coinciding with increased HCRT-1 leads to reductions in these behaviors. An explanation of this may be related to decreased sensitivity to CRH in lactating dams.

Although evidence supports the idea that HCRT and CRH show an excitatory interaction (Samson et al. 2002; Winsky-Sommerer et al. 2004), this was not observed for the lactating dams in the present study. These results may reflect the altered responsiveness of the HPA-axis and glucocorticoids in lactating dams. During the postpartum period, lactating rodents undergo many neuromorphological and neuroendocrine-related changes that promote adaptive responses to their offspring. Interestingly, lactating dams show increased basal levels of CRH mRNA in the
PVN (da Costa et al., 2001), and also show elevated ACTH and corticosterone in response to
suckling from pups (Voogt, Sar, & Meites, 1969; Lightman, 1992; Walker et al., 1992).
However, in response to acute stress challenges, the HPA system is downregulated in lactating
dams (da Costa et al., 1997; Walker et al., 1995; Lightman & Young, 1989), likely due to
increased activation of feedback loops from the adrenals (Lightman et al., 1990). The mice in
this study may have already been experiencing lactation-depended reduced CRH activity
following the introduction of a male intruder (acute stressor). This phenomenon may have
masked the effect of blocking CRH receptors on resulting behavior. When presented with an
intruder male, the dams in this study may not have responded in the predicted direction (as
indicated by increased aggression) to the CRH antagonist if they already have low CRH
responses to such a stressor. That is, the relative levels of this aggression may not be manipulated
in the expected direction of this study by blocking CRH receptors if the HPA system is already
down regulated. A CRH-receptor antagonist when given by itself does not alter maternal
aggression toward a male conspecific (Gammie et al., 2004). However, that study also showed
that CRH agonist administration leads to decreases in maternal aggression, as observed by
decreased number of attacks and duration of attacks and longer latencies to first attack a male
conspecific. Additionally, site-specific injections of CRH into the lateral septum also decreases
maternal defense (D’Anna & Gammie, 2009). HCRT-1 acts as an agonist to CRH (Samson et al.
2002), so one would expect that the CRH agonist would lead to decreased maternal aggression,
which has also been demonstrated (D’Anna & Gammie, 2006). Blocking CRH receptors as a
pretreatment to HCRT-1 at 0.3µg did not have any reinstating effects on maternal defense in the
present study. Although both CRH and HCRT-1 decrease maternal defense, they might be doing
so independently. According to the results of this study, it appears that the maternal deficits
related to elevated HCRT-1 are not indicative of an indirect effect of CRH disrupting maternal behavior, but rather, there is either a direct effect of HCRT-1 or perhaps an interaction with other factors that leads to the previously observed reductions in maternal responsiveness and maternal aggression. However, it is still possible that these two peptides are working in concert with one another to influence maternal aggression, especially after considering that blocking CRH along with increasing HCRT-1 activity led to differences in aggressive behavior (as opposed to giving a CRH antagonist alone and not observing any differences). Perhaps at optimal levels, these two peptides work together to promote an adaptive defensive response.

In addition to the lack of an effect of blocking CRH on rescuing maternal defense behavior, there was also no rescuing effect on pup-directed behaviors. No significant effect of CRH antagonist was observed on any of the measures, although there was a strong trend for the antagonist to decrease nursing behavior. The possibility that blocking CRH as a pretreatment to elevating HCRT-1 levels may lead to decreased nursing is interesting. Nursing is perhaps the most important maternal behavior, as there are two components to nursing that are important for pups: (1) providing food and (2) warmth. The pups are born altricial, such that they cannot feed themselves or regulate their body heat, thus requiring external care by the mother. Arched-back nursing allows the pups to gain access to food from the mother while at the same time keeps pups warm as the dam blankets her offspring. The dose of HCRT-1 used in this study (0.3µg) has been shown to decrease nursing behavior by decreasing total time spent nursing and increasing the time it takes for dams to begin to nurse (D’Anna & Gammie, 2006). Here, before administering HCRT-1 at the same dose, there was a trend for nursing to be decreased when blocking CRH, which may indicate an interesting dynamic between CRH and HCRT-1, one that
was not predicted *a priori* to this study. Although this was a nonsignificant trend, the results may warrant further exploration of how these two peptides interact in terms of maternal behavior.

Considering that there was no clear rescuing effect of blocking CRH receptors on pup-directed maternal behavior prior to administering HCRT-1 (0.3µg), it may not be surprising that HCRT-1 injections at this dose did not alter cellular activity in the BNST, PVN or lateral septum in our lactating dams. This result might suggest an overall reduced sensitivity to CRH during lactation or that HCRT-1 was not decreasing the behavior via these CRH rich areas, where both HCRT receptor subtypes have been identified (Marcus et al., 2001). Another possibility is that HCRT may induce its effects on maternal behavior via other mechanisms, such as activation of oxytocin, which has been shown alter maternal aggression when injected into the BNST (Consiglio, Borsoi, Pereira, & Lucion, 2005). However, there were some differences in maternal defense that were dependent on dose of CRH antagonist, and thus, we might expect to observe brain differences in response to HCRT-1. Differences in c-Fos expression might have been observed had we randomly primed some of these HCRT-1 injections with the CRH antagonist at 5.0µ on the days of brain removal and fixation. By doing so we could have seen how behavioral changes related to the doses of the CRH antagonist and HCRT-1 correspond with c-Fos activity. Furthermore, one of the caveats of single-labeling of c-Fos is that we cannot know how many of the cells stained are actually CRH-producing, which would need to be established in future studies utilizing a double-labeling procedure for c-Fos and CRH. CRH-producing neurons may have HCRT receptors, as observations of CRH neuronal excitation via HCRT-1 incubations would suggest (Samson et al., 2002). Additionally, HCRT-1 has been shown to produce increased activity of CRH producing neurons in areas like the PVN and central amygdala (Sakamoto et al., 2004), yet in this study there were no observed differences in CRH neuron-rich
areas, including the PVN, BNST, and lateral septum. As previously discussed, lactating dams show decreased response to HPA-axis agonistic influences (such as environmental stress or direct injections of glucocorticoids), and thus it is plausible that the missing effect of HCRT-1 activating CRH neurons observed in this study is due to reduced CRH sensitivity during the early postpartum period. It is also possible that elevating HCRT-1 at relatively high levels might change this dynamic in CRH sensitivity, such that blocking CRH leads to further decreases in adaptive responses. Further studies exploring this dynamic between HCRT and CRH postpartum might help to explain how rescuing effects of CRH antagonist were observed using a similar paradigm (CRH antagonist + HCRT-1) in other studies that used non-lactating dams (Ida et al., 2000a; Ida et al., 2000b), whereas in this study, blocking CRH led to decreased maternal defense and a trend for decreased nursing. It might be wise to employ CRH-c-Fos double-label immunohistochemistry post-administration to αhCRH vs saline + HCRT-1 (0.3µg) in the future to help neuroanatomically identify the source of this possible decrease in nursing and defense behavior.

There are several limitations to the study. First, the sample size was small, as the amount of maternal behavior and/or maternal defense behavior exhibited was low. I anticipated that approximately 90% of our animals would display aggression toward male intruders during the test, whereas only 58% of our dams displayed at least the minimum amount of aggression on at least one day of testing, which was an unexpected observation. Therefore, a large proportion of our animals did not display an adequate amount of aggression to be included in the study. This may have occurred for a few reasons related to the logistics of our housing situation. First, our animals are housed in an auto-flow cage apparatus, which produces a constant uncontrollable white noise. These cage systems may have produced a stressed state in our mice as a result (De
Boer, Van Der Gugten, & Slangen, 1998), which may have lead to a reduction in maternal defense behavior overall. Second, animals were transferred from homeroom to the surgery room for injections, and then transferred to the behavior testing room, which did not allow for habituation to the testing room. However, all of these procedures have been used in previous studies with no effect on defense behavior (Gammie et al., 2004; D’Anna & Gammie, 2006). Third, males and females are all housed in the same vivarium room of our facility. Mice have very strong olfactory senses, and therefore, our dams may have habituated to the scent of our virgin male intruders, which may have lead to a reduced aggressive response overall. All of this taken together could have produced a floor effect, making it difficult for us to make adequate comparisons between treatments. Another limitation could have been error in injection administration. The protocol for administering injections dictates that the 1µl of drug be injected slowly over the course of 1 minute, checking to see if the bubble created between dH₂O and drug moved in the direction of the injection. For most of these injections the bubble was not displaced at the end of the 1-minute interval, and could have created inconsistency in how the drug was administered, increasing variability in our sample. However, differences between drug conditions during the maternal aggression tests were still detected. These differences observed were in the opposite direction as predicted, as increased blocking of CRH led to decreases in frequency to attack the male intruder, as well as decreased tail rattling. Of course the third possibility is that the HCRT-1 agonist consistently reduced our animal’s defense behavior across all conditions as intended, and blocking CRH receptors does not rescue the behavior. Finally, it should be noted that the neuroanatomical portion of this study was underpowered, and thus, the lack of any observable differences in c-Fos expression between HCRT-1- and saline-injected animals might be due to the lack of sufficient power.
I hypothesized that pup-directed maternal behaviors would be rescued on the days that dams received the CRH antagonist, as this would block the effect of HCRT-1 acting on CRH receptors. This is not what my analysis showed. Many of the pup-directed behaviors in general were quite low compared to previous work on maternal behavior with the same strain of mouse, as latency to retrieve pups, to be on nest, and to begin nursing were quite large, and duration of nest building and time spent nursing were relatively low (D’Anna & Gammie, 2006). Therefore, it is possible that we experienced a floor effect in maternal behavior, which may have drowned out any effect of our drug, as the CRH antagonist simply did not rescue these behaviors; the dose of HCRT-1 used in this study overall lead to relatively lower levels of pup-directed maternal behavior. On the other hand, aggression levels were comparable to levels displayed in dams receiving the 0.3µg dose of HCRT-1 as observed by D’Anna and Gammie (2006), and therefore, the results for aggression might suggest that blocking CRH simply does not rescue defensive behaviors, but rather, may inhibit them further. It is possible that we blocked too much CRH for the dams to engage the male when it was placed in the dam’s cage. This would suggest that an inverted-U relationship occurs with CRH, such that at relatively low or high levels of CRH activity aggression behavior will be impaired, and furthermore, this might be occurring independently of HCRT-1 activity.

To date, this is the first and only study to investigate the interaction between HCRT and CRH under the context of maternal behavior. HCRT and CRH influence maternal behavior in profoundly similar ways, although the extent to which they interact with one another in terms of maternal behavior is still underexplored. Based on the results here, HCRT-1 might be affecting pup-directed behaviors and maternal defense independently of CRH, and reducing CRH activity may further reduce these behaviors, especially nursing and number of attack bouts. Considering
the strong anatomical connections, and reciprocal excitatory effects on one another’s nuclei between HCRT and CRH producing neurons, further exploration of how these two peptides work together to regulate aspects of maternal defense and pup-directed maternal behavior is warranted.
References


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Table 1. Friedman's Rank Test Across Drug Treatment for Maternal Behavior. Descriptive statistics for all behavioral measures used on the maternal behavior test (n = 11). Statistical procedure used was Friedman’s Rank test for dependent samples. There were no significant main effects observed across CRH antagonist treatments.

<table>
<thead>
<tr>
<th>Behavior in Seconds</th>
<th>Treatment prior to HCRT-1 (.3µg) Administration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ahCRH (2.5µg)</td>
<td>ahCRH (2.5µg)</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>On Nest</td>
<td>558.18</td>
<td>634.91</td>
</tr>
<tr>
<td>Off Nest</td>
<td>1232.73</td>
<td>627.18</td>
</tr>
<tr>
<td>Nursing</td>
<td>322.36</td>
<td>414.34</td>
</tr>
<tr>
<td>Lick/Groom (L/G)</td>
<td>19.55</td>
<td>17.78</td>
</tr>
<tr>
<td>Self-Groom</td>
<td>231.73</td>
<td>126.03</td>
</tr>
<tr>
<td>Nest Build (NB)</td>
<td>1.55</td>
<td>5.13</td>
</tr>
<tr>
<td>Pup Retrieval (PR)</td>
<td>12.91</td>
<td>13.35</td>
</tr>
<tr>
<td>Latency On Nest</td>
<td>812.82</td>
<td>864.04</td>
</tr>
<tr>
<td>Latency PR1</td>
<td>678.36</td>
<td>889.48</td>
</tr>
<tr>
<td>Latency PR4</td>
<td>1160.18</td>
<td>806.91</td>
</tr>
<tr>
<td>Latency PR All</td>
<td>1517.09</td>
<td>633.39</td>
</tr>
<tr>
<td>Latency NB</td>
<td>1511.00</td>
<td>649.84</td>
</tr>
<tr>
<td>Latency Nurse</td>
<td>1281.55</td>
<td>533.00</td>
</tr>
<tr>
<td>Latency L/G</td>
<td>690.09</td>
<td>670.69</td>
</tr>
</tbody>
</table>
Figure 1. Coronal section displaying a successful cannula placement into the lateral ventricles.
Figure 2. Mean comparisons of latency to first attack on male intruder during the maternal defense test ($n = 7$). Statistical procedure used was Friedman’s Rank test for related samples to test for main effects of CRH antagonist. No significant differences were observed. Error bars represent ± 1 SEM.
Figure 3. Mean comparisons of total duration of attack on male intruder during the maternal defense test ($n = 7$). Statistical procedure used was Friedman’s Rank test for related samples to test for main effects of CRH antagonist. No significant differences were observed. Error bars represent ± 1 SEM.
Figure 4. Mean comparisons of total duration of self-grooming during the maternal defense test ($n = 7$). Statistical procedure used was Friedman’s Rank test for related samples to test for main effects of CRH antagonist. No significant differences were observed. Error bars represent ± 1 SEM.
Figure 5. Mean comparisons of frequency of attacks on male intruder during the maternal defense test (n = 7). Statistical procedure used was Friedman’s Rank test for related samples to test for main effects of CRH antagonist. An overall main effect was observed (p = .044). Follow-up comparisons using Wilcoxon Signed-Rank test for related samples revealed significant differences between 2.5µg ahCRH and 5.0µg ahCRH ( * p < .05). Error bars represent ± 1 SEM.
Figure 6. Mean comparisons of total duration of tail rattling during the maternal defense test ($n = 7$). Statistical procedure used was Friedman’s Rank test for related samples to test for main effects of CRH antagonist. An overall main effect was observed ($p = .031$). Follow-up comparisons using Wilcoxon Signed-Rank test for related samples revealed significant differences between 2.5µg ahCRH and 5.0µg ahCRH ( * $p < .05$). Error bars represent ± 1 SEM.
Figure 7. c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN) for animals treated with HCRT-1 (0.3 µg) and saline. A, Mean comparisons of c-Fos expression in the PVN between HCRT-1 (n = 4) and saline (n = 8) treated animals. Mann-Whitney U test revealed no significant effect of HCRT-1 treatment on resulting c-Fos expression in the PVN (z = -1.021, p = .307). Error bars represent ± 1 SEM. B & C, c-Fos expression in the PVN for HCRT-1 (0.3 µg) and saline, respectively.
Figure 8. c-Fos expression in the bed nucleus of the stria terminalis (BNST) for animals treated with HCRT-1 (0.3µg) and saline. A, Mean comparisons of c-Fos expression in the BNST between HCRT-1 (n = 4) and saline (n = 8)treated animals. Mann-Whitney U test revealed no significant effect of HCRT-1 treatment on resulting c-Fos expression in the BNST (z = -1.108, p = .268). Error bars represent ± 1 SEM. B & C, c-Fos expression in the BNST for HCRT-1 (0.3µg) and saline, respectively.
Figure 9. c-Fos expression in the lateral septum area 1 (LS1) for animals treated with HCRT-1 (0.3µg) and saline. A, Mean comparisons of c-Fos expression in the LS1 between HCRT-1 ($n = 4$) and saline ($n = 8$) treated animals. Mann-Whitney U test revealed no significant effect of HCRT-1 treatment on resulting c-Fos expression in the LS1 ($z = -.340, p = .734$). Error bars represent ± 1 SEM. B & C, c-Fos expression in the LS1 for HCRT-1 (0.3µg) and saline, respectively.
Figure 10. c-Fos expression in the lateral septum area 2 (LS2) for animals treated with HCRT-1 (0.3µg) and saline. A, Mean comparisons of c-Fos expression in the LS2 between HCRT-1 ($n = 4$) and saline ($n = 8$) treated animals. Mann-Whitney U test revealed no significant effect of HCRT-1 treatment on resulting c-Fos expression in the LS2 ($z = 0.000$, $p = 1.000$). Error bars represent ± 1 SEM. B & C, c-Fos expression in the LS2 for HCRT-1 (0.3µg) and saline, respectively.
Figure 11. c-Fos expression in the lateral septum area 3 (LS3) for animals treated with HCRT-1 (0.3 µg) and saline. A, Mean comparisons of c-Fos expression in the LS3 between HCRT-1 (n = 4) and saline (n = 8) treated animals. Mann-Whitney U test revealed no significant effect of HCRT-1 treatment on resulting c-Fos expression in the LS3 (z = -1.189, p = .234). Error bars represent ± 1 SEM. B & C, c-Fos expression in the LS1 for HCRT-3 (0.3 µg) and saline, respectively.