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# CORTICOTROPIN-RELEASING-HORMONE RECEPTORS IN THE MEDIAL PREFRONTAL CORTEX REGULATE HYPOTHALAMIC-PITUITARY-ADRENAL ACTIVITY AND ANXIETY-RELATED BEHAVIOR REGARDLESS OF PRIOR STRESS EXPERIENCE

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## Abstract

The hypothalamic-pituitary-adrenal (HPA) axis habituates, or gradually decreases its activity, with repeated exposure to the same stressor. During habituation, the HPA axis likely requires input from cortical and limbic regions involved in processing of cognitive information that is important in coping to stress. Brain regions such as the medial prefrontal cortex (mPFC) are recognized as important in mediating these processes. The mPFC modulates stress-related behavior and some evidence suggests that the mPFC regulates acute and repeated stress-induced HPA responses. Interestingly, corticotropin releasing hormone(CRH)-1 receptors, which integrate neuroendocrine, behavioral and autonomic responses to stress, are localized in the mPFC but have not been specifically examined with respect to HPA regulation. We hypothesized that CRH receptor activity in the mPFC contributes to stress-induced regulation of HPA activity and anxiety-related behavior, and that CRH release in the mPFC may differentially regulate HPA responses in acutely- compared to repeatedly-stressed animals. In the present experiments, we found that blockade of CRH receptors in the mPFC with the non-selective receptor antagonist, D-Phe-CRH (50ng or 100ng) significantly inhibited HPA responses compared to vehicle regardless of whether animals were exposed to a single, acute 30min restraint or to the eighth 30min restraint. We also found that intra-mPFC injections of CRH (20ng) significantly increased anxiety-related behavior in the elevated plus maze in both acutely- and repeatedly-restrained groups compared to vehicle. Together, these results suggest an excitatory influence of CRH in the mPFC on stress-induced HPA activity and anxiety-related behavior regardless of prior stress experience.

### Keywords

prefrontal cortex; corticotropin releasing hormone; restraint; anxiety; ACTH; corticosterone

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## 1. Introduction

The medial prefrontal cortex (mPFC) has received considerable attention for its role in working memory, learning, attention and emotional behavior (Ragozzino, 2000; Birrell & Brown, 2000; Ridderinkhof et al, 2004) and is a site that is thought to perform the complex appraisals necessary to process the nature of aversive stimuli such as stressors (Quirk & Gehlert, 2003; Amat et al, 2005). There is substantial evidence that the mPFC influences activity in the main neuroendocrine system of the stress response, the hypothalamic-pituitary-adrenal (HPA) axis (Diorio et al, 1993; Sullivan & Gratton, 1999; Akana et al, 2001; Sullivan & Gratton, 2002; Figueiredo et al, 2003; Rangel et al, 2003; Spencer et al, 2005). Lesions of the mPFC potentiate HPA responses to processive (psychological/emotional) stressors such as restraint but often have no effect on responses to systemic stressors that represent an immediate threat to physiological homeostasis such as ether exposure (Diorio et al, 1993; Figueiredo et al, 2003). There are, however, exceptions to the latter case in which mPFC lesions have been reported to potentiate HPA responses to an immune challenge (Crane et al, 2003). Furthermore, the mPFC is sensitive to repeated stress (Bagley and Moghaddam, 1997; Finlay et al, 1997; Sullivan & Gratton, 1999; Akana et al, 2001). For example, acute tail shock elicits a two-fold greater increase in extracellular norepinephrine in the mPFC of repeatedly cold-stressed rats than in naive control rats (Finlay et al, 1997). Corticosterone implanted into the mPFC also inhibits ACTH responses to restraint in repeatedly, as well as acutely, cold-stressed rats (Akana et al, 2001). Importantly, mPFC lesions suppress restraint-induced corticosterone responses in repeatedly-, but not acutely-restrained rats (Sullivan and Gratton, 1999). The lack of effects in acutely-restrained rats in the latter study contradict the lesion studies described above that found that mPFC lesions potentiate HPA responses to acute restraint (Diorio et al, 1993; Figueiredo et al, 2003). This discrepancy may be due to differences in the size of mPFC lesions. Lesions in Sullivan & Gratton (1999) were relatively extensive and were observed in the entire mPFC including cingulate, prelimbic and infralimbic regions. Lesions in Diorio et al (1993) and Figueiredo et al (2003) were smaller and more confined to prelimbic and infralimbic regions. Together, the evidence discussed above suggests that activity in the mPFC regulates both acute and repeated stress-induced HPA activity. Repeated exposure to the same, homotypic stressor can produce a gradual decrement, or habituation, of HPA activity (Bhatnagar & Meaney, 1995; Li & Sawchenko, 1998; Garcia et al, 2000; Viau and Sawchenko, 2002; Jaferi & Bhatnagar, 2006). Habituation to repeated stress may involve discrimination of the stressor as a familiar and previously encountered stressor as opposed to one that is novel. During this process, the hypothalamic paraventricular nucleus (PVN) likely requires input from cortical and limbic regions involved in cognitive processing such as the mPFC (Melia et al, 1994; Cullinan et al, 1996). In addition to regulating HPA responses to stress, the mPFC also modulates anxiety-related behaviors. In particular, mPFC lesions generally decrease anxiety-related behavior as shown by increased time spent in the open arms of the elevated plus maze (Gonzalez et al, 2000; Lacroix et al, 2000; Shah & Treit, 2003), increased social interaction (Gonzalez et al, 2000; Shah & Treit, 2003), increased open field exploration (Lacroix et al, 2000), and lower rates of burying in the shock probe burying test (Shah & Treit, 2003). These lesion studies suggest that, normally, stimulation of the mPFC increases anxiety-related behavior. However, little is known about the specific neurotransmitters that mediate mPFC regulation of anxiogenic behavior or regulation of HPA activity.

Interestingly, corticotropin-releasing-hormone (CRH)-1 receptors, which play an intricate role in integrating neuroendocrine, behavioral and autonomic responses to stress (Bale & Vale, 2004), are found in large densities in the mPFC as demonstrated by immunohistochemistry and *in situ* hybridization (Radulovic et al, 1998; Van Pett et al, 2000). The presence of CRH-2 receptors has not been reported in the rodent mPFC (Chalmers et al, 1995). CRH stimulates HPA responses to a variety of acute stressors (Deak et al, 1999; Habib et al, 2000; McElroy et al, 2002; Rivier et al, 2003). In addition, CRH also plays a prominent role in inducing anxiety-

related behavior (Landgraf, 2001). Intracerebroventricular administration of CRH reduces open arm exploration on the elevated plus maze (EPM) (Baldwin et al, 1991; Adamec & McKay, 1993) and also has anxiogenic effects in other common tests of anxiety (Dunn & File, 1987; Takahashi et al, 1989). These anxiogenic-type effects of CRH appear to be mediated by the CRH-1 receptor subtype in particular (Heinrichs et al, 1997). However, whether CRH release *in* the mPFC regulates HPA activity or anxiety-related behavior has not been studied. In the present studies, we examined HPA responses to acute restraint or behavior in the elevated plus maze after CRH receptor blockade in the mPFC. We also assessed these measures in repeatedly-stressed animals because repeatedly, cold-stressed rats exhibit sensitized norepinphrine release in the mPFC after intraventricular CRH compared to acutely-stressed rats (Finlay et al., 1997), suggesting that the mPFC may be sensitized to CRH in repeatedly-stressed rats. In addition, activity in the mPFC is important for repeated stress-induced HPA activity, as described above. Therefore, in the first experiment, we hypothesized that CRH receptor activation in the mPFC stimulates HPA responses to acute and repeated stress, and that the actions of CRH at the mPFC on HPA activity will be of a greater magnitude in repeatedly-stressed animals. We tested this hypothesis in the first experiment by examining HPA responses to the 1$^{st}$ or 8$^{th}$ restraint exposure after a single intra-mPFC injection of the non-selective CRH receptor antagonist, [DPhe12, Nle21,38, CalphaMe Leu37] r/h CRH(12–41) (D-Phe-CRH). In the second experiment, we hypothesized that CRH receptor activity in the mPFC would increase anxiety-related behavior in both acutely- and repeatedly-stressed animals and that the effects of CRH in the mPFC will be of a greater magnitude in repeatedly-stressed animals. To test this hypothesis, we measured behavior in the EPM after intra-mPFC injections of vehicle or CRH in animals that were previously exposed to one or eight days of restraint.

## 2. Results

### Experiment 1a: Effect of intra-mPFC injections of 50ng of D-Phe-CRH on HPA responses to acute or repeated restraint

Cannula were stereotaxically localized to the mPFC and confirmed for correct placement as shown in Figure 1. Plasma ACTH responses to the 1$^{st}$ or 8$^{th}$ restraint after intra-mPFC injections of 50ng of D-Phe-CRH or vehicle are shown in Figure 2. At 0, 15 and 30 min, no significant effects on ACTH were observed. At 30 min, although there was a trend towards a Stress Group × Drug Treatment Interaction ($F(1,41)=3.6$, $p=0.06$), this effect was not significant. At 60 min, no significant effects on ACTH were observed.

Plasma corticosterone responses to the 1$^{st}$ or 8$^{th}$ restraint after intra-mPFC injections of 50ng of D-Phe-CRH or vehicle are also shown in Figure 2. No significant effects were observed at 0 min. At 15 min, there was a significant Stress Group effect ($F(1,50)=26.9$, $p\leq0.001$) and a significant Drug Treatment effect ($F(1,50)=12.4$, $p\leq0.001$). Similarly, at 30 min, there was a significant Stress Group effect ($F(1,45)=19.2$, $p\leq0.001$) and a significant Drug Treatment effect ($F(1,45)=10.2$, $p\leq0.01$). At 60 min, we observed a significant Stress Group effect ($F(1,41)=5.9$, $p\leq0.01$). For significant Stress Groups effects at 15, 30 and 60 min, repeatedly-restrained groups had lower corticosterone than acutely-restrained groups regardless of drug treatment. For significant Drug Treatment effects at 15 and 30 min, groups that received D-Phe-CRH treatment had lower corticosterone than vehicle groups regardless of whether or not they were repeatedly-stressed.

To summarize, repeatedly-restrained groups overall displayed significantly lower corticosterone responses to restraint than acutely-restrained groups at 15, 30 and 60 min (regardless of Drug treatment), providing evidence of habituation. D-Phe-CRH treated groups overall displayed significantly lower corticosterone than vehicle-treated groups at 15 and 30 min (regardless of stress group assignment).

### Experiment 1b: Effect of intra-mPFC injections of 100ng of D-Phe-CRH on HPA responses to acute or repeated restraint

Plasma ACTH responses to the 1st or 8th restraint after intra-mPFC injections of 100ng of D-Phe-CRH or vehicle are shown in Figure 3. No significant effects on ACTH were observed at 0 min. At 15 min, there was a significant Stress Group effect ($F(1,36)=15.8$, $p\leq0.001$). At 30 min, there was a significant Stress Group effect ($F(1,29)=10.4$, $p\leq0.01$) and a significant Drug Treatment effect ($F(1,29)=4.9$, $p\leq0.05$) in which groups that received D-Phe-CRH injection had lower ACTH than vehicle-injected groups. At 60 min, we observed a significant Stress Group effect ($F(1,34)=9.7$, $p\leq0.01$). For significant Stress Group effects at 15, 30 and 60 min, repeatedly-restrained groups had lower ACTH than acutely-restrained groups regardless of drug treatment.

Plasma corticosterone responses to the 1st or 8th restraint after intra-mPFC injections of 100ng of D-Phe-CRH or vehicle are also shown in Figure 3. No significant effects on corticosterone were observed at 0 min. At 15 min, there was a significant Stress Group effect ($F(1,35)=6.9$, $p\leq0.01$). At 30 min, we observed a significant Stress effect ($F(1,30)=35.1$, $p\leq0.001$) and a significant Drug Treatment effect ($F(1,30)=5.6$, $p\leq0.05$) in which groups that received D-Phe-CRH treatment had lower corticosterone than vehicle-injected groups. At 60 min, there was a significant Stress Group effect ($F(1,33)=12.1$, $p\leq0.001$). For significant Stress Group effects at 15, 30 and 60 min, repeatedly-restrained groups had lower corticosterone than acutely-restrained groups.

To summarize, repeatedly-restrained groups overall displayed significantly lower ACTH and corticosterone responses to restraint than acutely-restrained groups at 15, 30 and 60 min (regardless of drug treatment), providing evidence of habituation. D-Phe-CRH treated groups overall displayed significantly lower ACTH and corticosterone than vehicle-treated groups at 30 min regardless of whether or not they were repeatedly-stressed.

### Experiment 2: Effect of intra-mPFC injection of CRH on anxiety-related behavior in the elevated plus maze after acute or repeated restraint

Anxiety-related behaviors in the elevated plus maze after intra-mPFC injection of vehicle or 20ng CRH in acutely- and repeatedly-restrained rats are shown in Figure 4. No significant Stress Group effects were observed on any of the behaviors measured. There were significant Drug Treatment effects on the number of open arm entries ($F(1,23)=4.5$, $p\leq0.05$) and the percentage of total time spent in the open arms ($F(1,23)=5.0$, $p\leq0.05$) in which CRH-treated groups had fewer open arm entries and spent less time in the open arms compared to vehicle-treated groups regardless of stress condition. We also observed a significant Drug Treatment effect on the percentage of total time spent in the closed arms ($F(1,23)=5.5$, $p\leq0.05$) in which CRH-treated groups spent more time in the closed arms than vehicle-treated groups.

To summarize, overall, CRH administration in the mPFC significantly decreased the number of open arm entries as well as the percentage of total time spent in the open arms and significantly increased the percentage of total time spent in the closed arms compared to vehicle treatment.

## 3. Discussion

In the present studies, repeatedly-restrained rats in each experiment exhibited lower ACTH and/or corticosterone responses at 15 and/or 30min to the 8th restraint compared to acutely-restrained rats, providing clear evidence of habituation of HPA activity. However, the effects of CRH receptor blockade with either the 50 or 100ng dose of D-Phe-CRH were not specific to the repeated stress state. We found that CRH receptor blockade in the mPFC by the lower

dose (50ng) of D-Phe-CRH significantly inhibited corticosterone responses to restraint overall in both acutely- and repeatedly-stressed groups at 15 and 30 minutes, but did not significantly affect ACTH. A higher dose (100ng) of D-Phe-CRH significantly inhibited both ACTH and corticosterone responses overall in all animals at 30 minutes. Assessment of behaviors in the elevated plus maze showed that intra-mPFC injections of CRH in both acutely- and repeatedly-restrained animals significantly decreased the percentage of time spent in the open arms as well as the number of open arm entries and significantly increased the percentage of time spent in the closed arms. These behaviors are considered to be indices of increased anxiety (Pellow et al, 1985; Pellow & File, 1986). Therefore, the present studies demonstrate a role for CRH in the mPFC in increasing HPA responses to restraint and anxiety-related behavior regardless of prior stress experience.

In Experiment 1, blockade of CRH receptors in the mPFC with either 50ng or 100ng of D-Phe-CRH produced an inhibition of ACTH and/or corticosterone responses, depending on the experiment, to acute and repeated restraint. These results suggest that, normally, CRH acting in the mPFC exerts an excitatory influence on HPA responses to acute and repeated stress. Sources of stress-related afferent input to the mPFC are numerous and include the hippocampal formation, basolateral amygdala and paraventricular thalamus (Sarter & Markowitsch, 1983; Conde et al., 1995; Pinto et al., 2003; Jaferi and Bhatnagar, 2006). These inputs provide the mPFC with cognitive and emotionally salient information related to the stressor (Herman et al., 1996) in addition to memories related to past stress experiences (Roozendaal et al, 2004). Although the mPFC does not directly innervate the PVN, it can modulate HPA activity through its connections to the bed nucleus of the stria terminalis (BNST) or peri-PVN, important afferent sites of the PVN (Swanson & Sawchenko, 1983; Roland & Sawchenko, 1993). The mPFC could also regulate HPA activity through regions such as the basolateral and central amygdala that project indirectly to the PVN through the BNST (Dong et al, 2001). Regardless of the path by which CRH release in the mPFC regulates HPA activity, the present data demonstrate that acute restraint-induced CRH release in the mPFC normally excites HPA activity.

In the present studies, the majority of our bilateral cannula placements were in the dorsal region of the mPFC although we also accepted one cannula in the dorsal mPFC and one cannula in the ventral mPFC (infralimbic). This is because it is highly likely that drug administered into the dorsal region also diffused into the ventral region. However, it is worth noting that evidence exists for neuroanatomical and functional differences between dorsal (prelimbic) versus ventral (infralimbic) subregions of the rodent mPFC (Heidbreder & Groenewegen, 2003). For example, the prelimbic subregion primarily projects to limbic sites that reportedly affect cognition (Vertes et al, 2004) while the infralimbic subregion projects extensively and directly to autonomic/visceral-related cell groups in the hypothalamus and brainstem (Terreberry & Neafsey, 1987; Hurley et al, 1991; Vertes et al, 2004). In addition to differences in neuroanatomical connectivity, differential functional contributions have been reported with respect to drug-induced behavioral sensitization (Tzschentke & Schmidt, 2000) and acquisition and extinction of conditioned fear (Morgan & LeDoux, 1995). Therefore, further investigation with more selective injections into the infralimbic mPFC, although difficult, may be useful in clearly determining whether CRH plays differential roles in the prelimbic versus infralimbic mPFC when regulating stress-induced HPA activity.

As mentioned above, the infralimbic mPFC projects directly to autonomic/visceral-related cell groups in the hypothalamus and brainstem that may play a role in modulating visceral responses to emotional stimuli (Terreberry & Neafsey, 1987; Hurley et al, 1991; Vertes et al, 2004). In turn, autonomic innervation of the adrenal gland can act as an extra-ACTH mechanism in regulation of adrenal corticosteroid secretion (Edwards & Jones, 1993; Engeland, 1998). This may explain the dissociation of ACTH and corticosterone secretion in Experiment 1a in which

D-Phe-CRH administration in the mPFC significantly altered stress-induced corticosterone without significantly altering ACTH levels. Manipulations of the infralimbic mPFC may indirectly alter adrenal functioning via its direct influence on autonomic-related neurons. The results of Experiment 1b, in which a higher dose of D-Phe-CRH (100ng) was used, differed from the results of Experiment 1a in that the drug did significantly alter both ACTH and corticosterone. There was, however, a more noticeable effect on ACTH responses to acute restraint than on corticosterone responses. Perhaps additional systems that influence the ACTH response to an acute stressor were affected by the higher dose of CRH receptor antagonism in the mPFC, but not by the lower dose, which resulted in a different HPA response in Experiment 1a versus 1b.

In Experiment 2, we found that intra-mPFC administration of CRH increased behaviors that are indicative of anxiety on the EPM. Furthermore, CRH in the mPFC produced increased anxiety-related behavior compared to vehicle treatment to a similar extent in animals that were exposed to 1 or 8 days of restraint prior to testing. These findings are in line with mPFC lesion studies that suggest that the mPFC normally acts to increase anxiety in the elevated plus maze (Gonzalez et al, 2000; Lacroix et al, 2000; Shah & Treit, 2003), the social interaction test (Gonzalez et al, 2000; Shah & Treit, 2003), the open field (Lacroix et al, 2000), and the shock probe burying test (Shah & Treit, 2003). However, to our knowledge, the present findings are the first to show that CRH receptor activation in the mPFC is important for increases in anxiety-related behavior in stressed animals. In contrast to the results of the lesion studies above, another mPFC-lesion study by Morgan & Ledoux (1995) suggested that the mPFC normally decreases anxiety-related behaviors in a fear conditioning paradigm (Morgan & Ledoux, 1995). An inhibitory influence on anxiety-related behavior might be mediated by neurotransmitters other than CRH such as GABA, opioids or dopamine, all of which decrease anxiety-related behavior as shown by selective manipulation of their receptors in the mPFC (Espejo, 1997; Wall & Messier, 2000; Shah et al, 2004.) With respect to characterizing the role of CRH in the mPFC on anxiety-related behavior, one caveat of the present study is that we used a single behavioral test for anxiety. Multiple behavioral tests will need to be employed in future studies in order to more thoroughly assess whether CRH in the mPFC has a uniformly excitatory effect on anxiety-related behaviors.

Interestingly, in Experiment 2, acute and repeated stress did not differentially impact anxiety-related behavior in the EPM in vehicle-treated groups. Previous reports on the effects of repeated restraint on anxiety-related behavior have yielded mixed results. For example, repeated restraint ranging from 10–21 days had no effect on anxiety-related behavior 2 hours later in the EPM (Thorsell et al, 1999; Chadda & Devaud, 2005), or 24 hours later in the open field test (Perrot-Sinal et al, 2004; Gregus et al, 2005) or the social interaction test (Gregus et al, 2005) compared to unstressed controls. However, another study that exposed rats to 14 days of restraint (2 or 8 hours/day) reported a significant decrease in the percentage of time spent in the open arms and number of open arm entries in the EPM compared to unstressed groups 48 hours following the last stress exposure (Kim & Han, 2006). The differences in the literature on the effects of repeated restraint on behavior in anxiety tests may reflect methodological variations in the duration of stress exposure and in the time points post-stress at which behavioral tests are conducted (Kim & Han, 2006). Many of the studies described above compare behavior of repeatedly-stressed groups to unstressed controls while we compared behavior of repeatedly-stressed rats to acutely-stressed rats. A single restraint reliably increases anxiety-related behavior in the EPM 24 hours later compared to unstressed controls (Mendonca & Guimaraes, 1998; Calvo & Volosin, 2001). Whether CRH in the mPFC regulates anxiety-related behavior in animals without any prior stress experience remains to be seen.

Although we did not assess CRH receptor binding and number in the mPFC in these experiments, the existing literature does suggest that CRH receptors can change after both acute

and repeated stress in a number of brain regions, and that the direction of these changes is region-specific. For example, Makino et al (1995) reported that both acute (2hr) and repeated immobilization stress (2hr daily for 14 days) increased CRH receptor mRNA in the PVN, but decreased it in the anterior pituitary, and did not affect CRH receptor mRNA in the BLA. In another study, repeated social stress decreased the number of binding sites in the anterior pituitary and hippocampus, but increased the number of binding sites in the central and lateral amygdala as well as in various cortical regions including the frontal cortex and cingulate cortex (Fuchs & Flugge, 1995). Based on these studies, it appears that CRH receptor binding does not always remain stable with stress, and like other cortical regions, it is possible that the prelimbic and infralimbic mPFC might also display an increase in the number of CRH binding sites after repeated stress. However, even if there were changes in CRH receptor binding or number in the mPFC in our acutely- versus repeatedly-stressed groups, these changes were either not sufficient or relevant for producing differential HPA responses or anxiety-related behavior in acutely-versus repeatedly-stressed animals.

The lack of repeated stress-specific effects of CRH receptor manipulation in the mPFC, particularly with respect to HPA activity, were unexpected. Some evidence exists for a role for the mPFC in specifically regulating HPA responses in repeatedly-stressed rats. Lesions of the mPFC decrease restraint-induced corticosterone responses in repeatedly-restrained rats without affecting responses in acutely-restrained rats (Sullivan and Gratton, 1999). Furthermore, acute tail shock elicits a two-fold greater increase in extracellular norepinephrine in the mPFC of repeatedly cold-stressed rats compared to controls (Finlay et al, 1997). Glutamate in the PFC may also play a role in the neurochemical response to repeated stress since extracellular levels of glutamate in the mPFC decrease with repeated exposure to tail pinch (Bagley and Moghaddam, 1997). Additionally, the mPFC receives substantial input from the paraventricular thalamus (Pinto et al, 2003; Jaferi & Bhatnagar, 2006), a midline thalamic nucleus whose posterior division has been demonstrated to regulate HPA activity and anxiety-related behavior particularly in repeatedly-stressed animals (Bhatnagar et al, 2000, 2002, 2003). Therefore, it is possible that the mPFC receives information specific to the repeated stress state and integrates this information about prior experience with the appropriate behavioral and neuroendocrine outputs, but does this independent of CRH receptor activity.

In sum, the central findings of the present studies are that CRH receptor blockade in the mPFC inhibits HPA responses to both acute and repeated stress, and that CRH injections into the mPFC increase anxiety-related behavior in the elevated plus maze in both acutely- and repeatedly-stressed animals. These findings suggest that CRH acting at the mPFC increases stress-induced HPA activity and anxiety-related behavior regardless of prior repeated stress experience. The relevant sources of CRH to the mPFC remain to be determined. CRH inputs to the mPFC are known to derive from the laterodorsal tegmental nucleus of the pons, which contains cells that are co-localized for CRH and acetylcholinesterase (Crawley et al, 1985), as well as CRH-1 receptors and choline acetyltransferase (Sauvage & Steckler, 2001). Since cholinergic inputs to the mPFC influence attentional performance (Dalley et al, 2004), interactions between CRH and acetylcholine might influence attentional processes in stressed animals. Another possible source of CRH inputs to the mPFC is the locus coeruleus, an afferent site of the mPFC which contains an abundance of CRH-containing cell bodies (Swanson et al, 1983; Van Bockstaele et al, 1996), and plays substantial roles in behavioral responses to stressful stimuli (Smagin et al, 1996).

Additionally, intra-mPFC sources of CRH cannot be ruled out. In conclusion, the present experiments demonstrate an excitatory role for CRH receptor activation in the mPFC on stress-induced HPA activity and anxiety-related behavior regardless of whether or not animals have been exposed to prior repeated stress.

# 4. Methods

## Animals

All experiments used adult male Sprague-Dawley rats supplied by Charles River (Wilmington, MA). Body weights ranged from 220–250g upon arrival at the animal housing facilities at the Department of Psychology, University of Michigan (Experiments 1a, 2) or at the Joseph Stokes Research Institute at Children's Hospital of Philadelphia (Experiment 1b). Rats were individually housed in polypropylene tub cages lined with bedding material, and were allowed ad libitum access to rat chow and water. They were maintained on a 12 hr light/dark schedule (lights on at 07:00 h), and all experiments took place during the trough of the diurnal rhythm, starting at 10am. Animals were briefly handled the day before experiments were conducted. All experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan (Experiments 1a, 2) and by the Institutional Animal Care and Use Committee at the Joseph Stokes Research Institute (Experiment 1b).

## Intracerebral cannula implantation & drug injection

Rats were anesthetized with a mixture of ketamine, xylazine and acepromazine (77: 1.5: 1.5 mg/ml given i.m. at 0.1 ml/100g body weight) and placed in a stereotaxic apparatus with the skull flat, and the tooth bar at −3.3mm. Bilateral guide cannula (22 gauge) were lowered into the mPFC according to the following coordinates from bregma: AP: +3.2mm, ML: ±0.8mm, DV: −3.0mm. The selection of these coordinates was based on previous studies using cannula in the mPFC (Baldwin et al, 2002; Capriles et al, 2003). Cannulae were then cemented into place using dental cement anchored by skull screws. Dummy cannulae were used to close the guide cannulae. At the time of experimentation, the dummy cannula was removed and replaced with an injector cannula connected to PE tubing attached to a Hamilton syringe. Drug or vehicle was injected over a 1 minute period, with the needle remaining in place for another one minute before removal. The injection volume that we used (0.5ul) has previously been used for microinjection of a variety of drugs into the prelimbic and infralimbic mPFC (Morency et al, 1987; Amat et al, 2005). While every drug may have a distinct solubility and distribution when injected locally, it is of interest that this 0.5ul volume has been demonstrated by Shah & Treit (2004) to be limited to the mPFC when microinjecting the benzodiazepine, midazolam. Specifically, the authors used the Chicago blue stain to estimate the extent of diffusion of intra-mPFC infusions when using dorsal-ventral coordinates similar to our own. They illustrated that their infusions were primarily limited to the ventral prelimbic and infralimbic subregions of the mPFC.

## Repeated stress paradigm

We used a repeated restraint paradigm in order to examine habituation of HPA activity in experiment 1 and anxiety-related behavior in experiment 2. Restraint, which is considered to be a psychological or processive stressor (Herman et al, 1996), consisted of placing rats in a ventilated, cylindrical plexiglass tube for 30 min. Rats in acute stress groups were exposed to a single 30 min restraint. Rats in repeated stress groups were exposed to 8 consecutive days of 30 min restraint per day. We, and other groups, have observed habituation of ACTH and corticosterone responses following 30min of restraint per day for 8 days and up to14 days (Viau and Sawchenko, 2002; Jaferi et al, 2003; Jaferi & Bhatnagar, 2006).

## Experiment 1: Effect of CRH receptor blockade in the mPFC on HPA responses to acute or repeated restraint

In Experiment 1a, we injected vehicle or 50ng of D-Phe-CRH bilaterally into the mPFC. This dose has previously been used by others to examine the role of CRH receptors in stress-induced reinstatement of cocaine-seeking (Erb et al, 2001), relapse to alcohol (Le et al, 2002),

behavioral consequences of uncontrollable stress (Hammack et al, 2002), and stress-induced c-fos expression (Funk et al, 2003) after intracerebral administration. We found that 50ng of D-Phe-CRH had significant effects on corticosterone, but not on ACTH. Furthermore, the 50ng dose did not differentially affect responses to acute versus repeated restraint. Therefore, we subsequently tested 100ng D-Phe-CRH in Experiment 1b, a dose previously used for injections of D-Phe-CRH into the lateral septum to study stress-induced freezing (Bakshi et al, 2002). The following methods for Experiment 1a and 1b are identical with the exception of the dose of D-Phe-CRH used.

After 5–7 days of recovery from stereotaxic implantation of cannula in the mPFC, half of all rats were randomly assigned to either 1 or 8 days of 30 min restraint/day. On day 1 or day 8, rats received intra-mPFC injections of 0.5ul of D-Phe-CRH or vehicle 30 min prior to restraint and blood collection as described below. The 30 min post drug-injection time point has previously been used to examine footshock-induced defensive withdrawal as well as footshock-induced reinstatement of cocaine-seeking 30 min following local or intracerebrovetricular injection of D-Phe-CRH (Erb & Stewart, 2001; Bruijnzeel et al, 2001). Blood was collected according to the procedure described below. The final group sizes were n= 7–15 per time point for Experiment 1a, and n= 7–11 per time point for Experiment 1b. The *n*'s represent the final *n*'s after omission of rats with placements of cannula outside of the mPFC. To obtain the final *n*'s, Experiments 1a and 1b were each run in two separate batches. Placement criteria are described below.

### Blood sampling procedure

In experiment 1, we collected blood samples on day 1 or 8 of restraint for assessment of HPA activity. 30 minutes after an intra-mPFC injection of drug or vehicle, each animal was placed in the restrainer and blood samples were taken from the tail vein at 0, 15 and 30 min during restraint. After collection of the 30 min samples, animals were removed from their restrainers and replaced in their home cages. At 60 min, blood samples were collected again and animals subsequently returned to their home cages. All samples were collected within 60 seconds of opening the cage in order to ensure that ACTH and corticosterone levels in plasma do not rise in response to the restraint itself (Akana et al, 1996). This sampling method of tail nicking, used by other groups as well, produces little reactivity in the animal and results in consistent basal and stress levels of ACTH and corticosterone (Bhatnagar & Dallman, 1998; Bhatnagar et al, 2000; Vahl et al, 2005).

### Experiment 2: Effect of CRH administration in the mPFC on anxiety-related behavior following acute or repeated restraint

We examined whether bilateral injections of CRH in the mPFC alter anxiety-related behavior in the elevated plus maze in rats that are exposed to either 1 or 8 days of restraint. Intra-mPFC administration of CRH is only expected to activate the CRH-1 receptor subtype since the mPFC reportedly contains CRH-1, but not CRH-2, receptors (Chalmers et al, 1995; Radulovic et al, 1998; Van Pett et al, 2000). In addition, CRH has a relatively low affinity for the CRH-2 receptor (Bale & Vale, 2004). Before injecting CRH in acutely versus repeatedly restrained rats in this experiment, we first conducted a pilot study to determine a dose of CRH that would alter behavior in the EPM after acute restraint when injected into the mPFC (*n* =5–6; data not shown). We injected CRH (20ng, 200ng) or vehicle into the mPFC 30 min prior to testing. The selection of these doses was based on previous studies from our lab that injected CRH into the basolateral amygdala to examine behavior in the EPM and from others who have injected similar doses of CRH into the amygdala and examined behavioral activation and grooming in an open field (Wiersma et al, 1998; Daniels et al, 2004). We found that 20ng of CRH decreased the number of open arm entries (0.3 ± 0.2) compared to vehicle (2.8 ± 1.2) as well as the time spent in the open arms (2.7 sec ± 1.2) compared to vehicle (29.6 sec ± 10.7). For rats receiving

200ng, the number of open arm entries (2.4 + 1.3) and the time spent in the open arms (30.8 + 11.0) was similar to vehicle-treated rats. Therefore, we chose the 20ng dose of CRH in this experiment. It was surprising that a higher dose of CRH had no substantial effect on EPM behavior, but the lower dose did. CRH generally produces dose-dependent increases in anxiety-related behaviors when microinjected into limbic regions such as the BNST (Sahuque et al, 2006) or periaqueductal grey (Martins et al, 1997). However, very little is known about CRH receptor activation specifically in cortical regions and its effects on behavior. Perhaps, in the mPFC, CRH receptor activation above a certain level activates other mechanisms that serve to bring stress-induced anxiety levels back to normal. To fully characterize these potential dose-dependent effects on behavior, future studies would need to employ a range of doses with a larger number of animals than was used in our pilot study.

After 5 days of recovery from stereotaxic implantation of cannulae in the mPFC, half of all rats were randomly assigned to either 1 (acute stress rats) or 8 days of 30 min restraint/day (repeated stress rats). On day 2 or day 9, rats received 0.5ul of 20ng CRH or vehicle into the mPFC at 30 min prior to EPM testing (n=6–8). The *n*'s represent the final *n*'s after omission of rats with placements of cannula outside of the mPFC. To obtain the final *n*'s, experiments were run in two separate batches.

## Behavior in the Elevated plus maze

In Experiment 2, we conducted testing approximately 24 hours following 1st or 8th restraint. Evaluation of responses on the EPM 24 hours after restraint is a commonly used test of stress-induced anxiety-like behavior in rodents (Martijena et al, 1997; Mendonca & Guimaraes, 1998; Calvo and Volosin, 2001). We selected the EPM as our test of anxiety-related behavior because of its documented utility in measuring stress-induced anxiety following exposure to various stressors including restraint (Martijena et al, 1997; Mendonca & Guimaraes, 1998; Calvo and Volosin, 2001; Korte & De Boer, 2003) and because it does not require lengthy training, the use of noxious stimuli such as electric shock, or manipulation of appetitive behaviors. Furthermore, the EPM has been pharmacologically validated, as a test of anxiety in rodents (Pellow et al, 1985; Pellow & File, 1986). Rats confined to the open arms show significantly more behavioral and physiological indices of anxiety than rats confined to the closed arms as shown by increased freezing, immobility, defecation as well as increased corticosterone secretion (Pellow et al, 1985). Rats also spend more time in the closed arms than the open arms and enter them more frequently than the open arms regardless of factors relating to illumination level or novelty (Pellow et al, 1985). This aviodance of the open arms likely reflects the rodent's innate aversion to open spaces. These pharmacological, behavioral and physiological studies strongly suggest that the behavior measured in the EPM is a valid measure of anxiety-related behavior in rodents. The plus-shaped apparatus consisted of two open and two closed arms with an open roof, arranged such that the two arms of each type are opposite to each other. At the onset of testing, each rat was placed onto the central area of the plus maze facing one of the closed arms. Behavior for each rat was recorded for 10 minutes by a video camera facing the closed arm. The maze was cleaned with a bleach/water solution after each rat was tested. The following EPM behaviors were later scored by two experimenters, one of which was blind to drug/stress condition of the animal being analyzed: 1. total number of open arm entries (counted when all four paws are placed in the open arm), 2. time spent in open arms (with all four paws in open arms)/total time, 3. time spent in closed arms (with all four paws in closed arms)/total time.

## Confirmation of cannula placements

After completion of the experiment, brains were collected, post-fixed in 4% formalin and sliced coronally at 30um on a cryostat and mounted onto Superfrost Plus slides. The exact placement of the cannulae was confirmed by staining sections with cresyl violet and visualizing the tips

of the cannulae. Representative cannulae placements are shown in Figure 1. Any animals whose cannulae placements were found to be outside of the mPFC were excluded from the analysis. More specifically, animals were deemed to have inappropriate placements if one or both of the cannula were located outside of the dorsal mPFC (anterior cingulate and prelimbic subregion) and ventral mPFC (infralimbic subregion) as outlined by the atlas of Paxinos and Watson (1997). Out of the rats that were included in the analysis, 65% had both cannula placed in the dorsal mPFC in Experiment 1a, 100% had both cannula in the dorsal mPFC in Experiment 1b and 93% had both cannula in the dorsal mPFC in Experiment 2. The rest had both cannula in the infralimbic subregion, or one cannula in dorsal mPFC and the other in infralimbic mPFC. Given the likely diffusion of the drug into dorsal and ventral regions of the mPFC, it is reasonable to conclude that the observed drug effects are due to actions in both dorsal and ventral subregions of the mPFC.

### ACTH and Corticosterone Radioimmunoassays

Blood was collected in microcentrifuge tubes containing10ul of sodium EDTA and kept on ice until centrifuged. After centrifugation, the plasma was aliquoted and kept frozen at −20° Celsius until assay. Plasma ACTH was measured by using a specific antiserum generously donated by Dr. William Engeland (Univ. of Minnesota) at a final dilution of 1:120,000, and [I125]ACTH as tracer (Diasorin, Stillwater, MN). The minimum level of detection of the assay is 10 pg/ml. Plasma corticosterone was measured using a kit from MP Biomedicals (Irvine, CA) and its minimum detection level is 0.625 ug/dl.

### Drugs

In experiment 1, [DPhe12, Nle21,38, CalphaMe Leu37] r/h CRH(12–41) (D-Phe-CRH) (Bachem) was dissolved in 0.9% saline. In experiment 2, CRH (Sigma) was dissolved in 0.9% saline. 0.9% saline served as the vehicle for all experiments. D-Phe-CRH, CRH and vehicle solutions were prepared the day before intra-mPFC injections.

### Statistical Analyses

Data were analyzed using analysis of variance (ANOVA). In Experiment 1, Stress Group (acute or repeated stress) × Drug Treatment (vehicle or D-Phe-CRH, either 50ng or 100ng) ANOVAs were performed at each time point of blood sampling. Due to missing samples at certain time points because of insufficient blood collection or problems in the assays, we were unable to perform repeated measures ANOVAs in this experiment. In Experiment 2, Stress Group (acute or repeated stress) × Drug Treatment (vehicle or CRH) ANOVAs were carried out for behavior in the EPM. The significance levels in all tests were set at $p \leq 0.05$.

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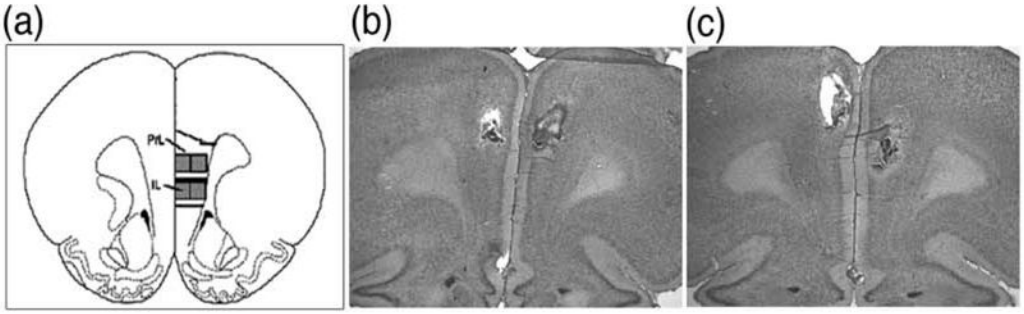


**Figure 1.**

Images of cannula placements in mPFC. The dorsal (prelimbic- PL) and ventral (infralimbic- IL) subregions of the mPFC (+3.2mm from bregma) based on the atlas of Paxinos and Watson are shown in a. Shown in b and c are representative cresyl violet-stained sections. Shown in b is an animal with the tracks of both injector cannulae placed in the dorsal mPFC (anterior cingulate, prelimbic). Shown in c is an animal with one cannula in the dorsal mPFC and other in the ventral mPFC (infralimbic). The majority of cannula placements in each experiment were bilaterally in the dorsal mPFC (65% in Experiment 1a; 100% in Experiment 1b; 93% in Experiment 2).

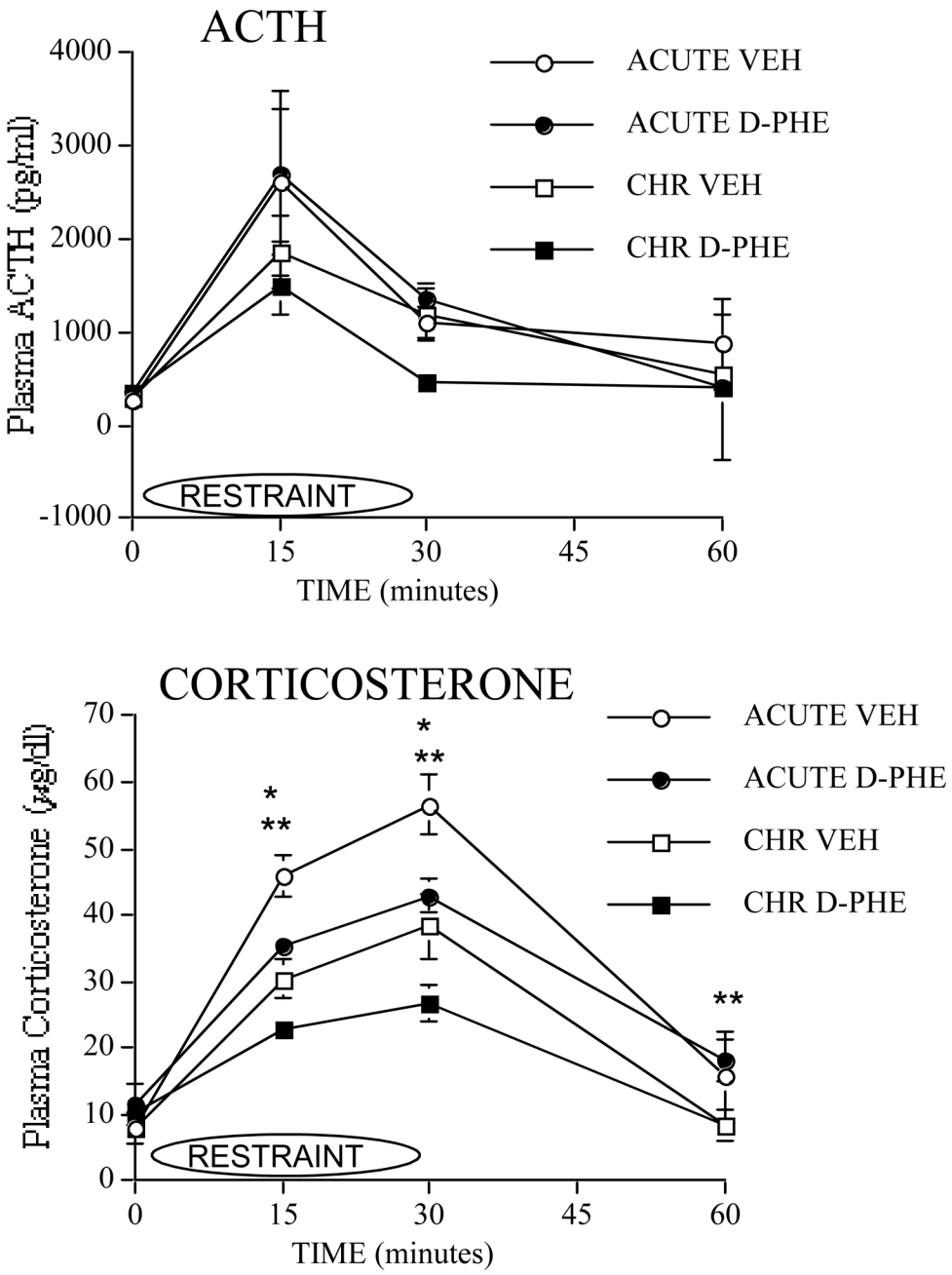


**Figure 2.**

Effect of 50ng of D-Phe-CRH in mPFC on ACTH and corticosterone responses to acute or repeated stress. Plasma ACTH and plasma corticosterone responses to the first restraint (acute restraint) or to the eighth restraint (repeated restraint) are shown in rats that received intra-mPFC injections of 50ng of D-Phe-CRH or VEH 30min prior to the 1st or 8th restraint. (The following are sample sizes at each time point of the ACTH response. For acute vehicle, n=8 at 0 min, n=9 at 15 min, n=11 at 30 min, and n=10 at 60 min. For acute drug, n=7 at 0 min, n=8 at 15 min, n=10 at 30 min, n=9 at 60 min. For repeated vehicle, n=7 at 0 min and 15 min, n=12 at 30 min, and n=10 at 60 min. Lastly, for repeated drug, n=8 at 0 min, n=7 at 15 min, n=12 at 30 min, and n=10 at 60 min. The following are sample sizes at each time point of the corticosterone response. For acute vehicle, n=11 at 0 min, n=14 at 15 min, n=13 at 30 min, and

n=7 at 60 min. For acute drug, n=11 at 0 min, n=14 at 15 min and 30 min, and n=10 at 60 min. For repeated vehicle, n=12 at 0 min, n=14 at 15 min, n=13 at 30 min, and n=14 at 60 min. Lastly, for repeated drug, n=8 at 0 min, n=12 at 15 min, n=9 at 30 min, and n=15 at 60 min.)
*D-Phe-CRH-injected groups are significantly lower than vehicle-injected groups regardless of whether animals were acutely or repeatedly stressed, reflecting a significant Main effect of Drug Treatment. $p \leq 0.05$.
** Repeatedly-stressed groups are significantly lower than acutely-stressed groups regardless of drug treatment, reflecting a significant Main effect of Stress Group, $p \leq 0.05$.

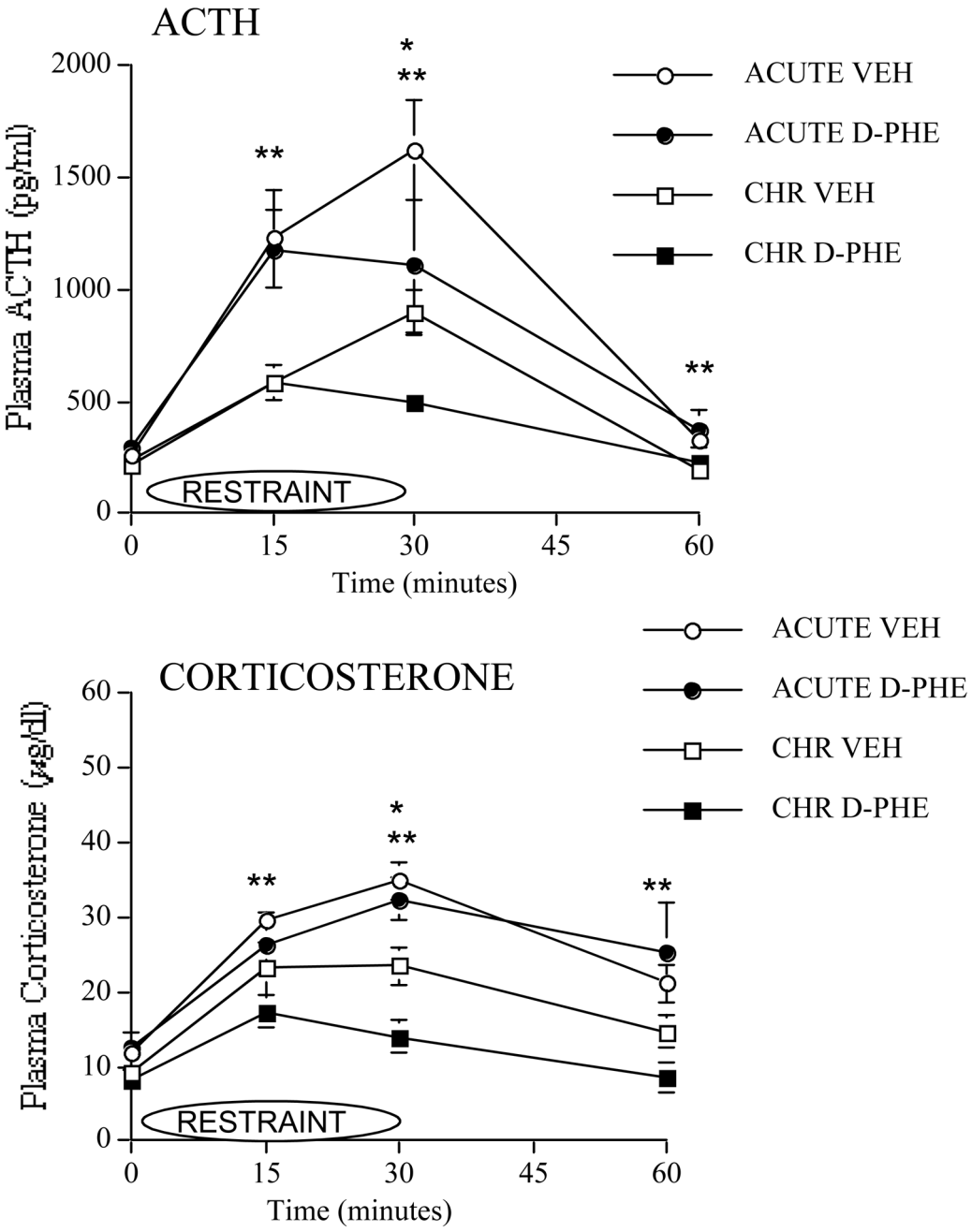


**Figure 3.**

Effect of 100ng of D-Phe-CRH in mPFC on ACTH and corticosterone responses to acute or repeated stress. Plasma ACTH and plasma corticosterone responses to the first restraint (acute restraint) or to the eighth restraint (repeated restraint) are shown in rats that received intra-mPFC injections of 100ng of D-Phe-CRH or VEH prior to day 1 or 8 of restraint. (The following are sample sizes at each time point of the ACTH response. For acute vehicle, n=11 at 0, 15 and 30 min, and n=10 at 60 min. For acute drug, n=10 at 0 min and 15 min, n=7 at 30 min, and n=9 at 60 min. For repeated vehicle, n=10 at 0 min and 15 min, n=7 at 30 min, n=9 at 60 min. Lastly, for repeated drug, n=10 at 0 min, n=9 at 15 min, n=8 at 30 min, and n=10 at 60 min. The following are sample sizes at each time point of the corticosterone response. For acute

vehicle, n=9 at 0 min, n=11 at 15 min, n=8 at 30 min, and n=10 at 60 min. For acute drug, n=7 at all time points. For repeated vehicle, n=11 at 0 min and 15 min, and n=9 at 30 min and 60 min. Lastly, for repeated drug, n=7 at 0 min, and n=10 at 15 min and 30 min, and n=11 at 60 min.)

* D-Phe-CRH-injected groups are significantly lower than vehicle-injected groups regardless of whether animals were acutely or repeatedly stressed, reflecting a significant Main effect of Drug Treatment, $p \leq 0.05$.

** Repeatedly stressed groups are significantly lower than acutely stressed groups regardless of drug treatment, reflecting a significant Main effect of Stress Group, $p \leq 0.05$.

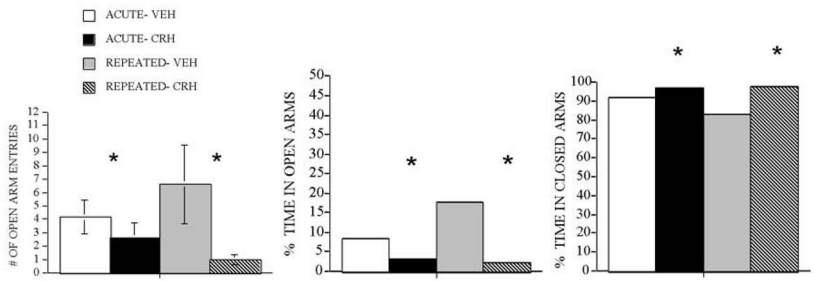


**Figure 4.**

Effect of administration of 20ng CRH in mPFC on behavior in elevated plus maze in acutely and repeatedly stressed animals. (a) number of open arm entries, (b) percentage of total time spent in open arms and (c) percentage of total time spent in closed arms in the elevated plus maze following intra-mPFC injection of 20ng of CRH or vehicle (VEH) are shown in rats previously exposed to 1 restraint (acute) or 8 restraints (repeated), n= 6–8.

* Animals injected with CRH in mPFC are overall significantly different from animals injected with VEH regardless of whether they were acutely or repeatedly stressed, reflecting a significant Main effect of Drug Treatment. $p \leq 0.05$.