CORTICOTROPIN RELEASING HORMONE RECEPTORS MODULATE STRESS-RELATED BEHAVIOUR THROUGH SEROTONIN VARIATIONS IN MOUSE BRAIN

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Table of Contents

Abstract:

Stressful events may be risk factors for anxiety and depression, possibly owing to variations of corticotropin-releasing hormone (CRH) receptors and monoamine activity. Moreover, the potential behavioural and cellular effects of CRH may involve activation of serotonin (5-HT) functioning. Although CRH actions in the hypothalamic-pituitary-adrenal (HPA) axis have been implicated in stress-related behaviour, it appears that extrahypothalamic regions may also be involved. Infusion of CRH into the central amygdala (CeA) or prefrontal cortex (PFC) produce anxiety-like behaviour that is reversible by CRH receptor antagonists. The actions of CRH may also involve indirect effects stemming from activation of receptors present on the dorsal raphe nucleus (DRN), leading to 5-HT variations in brain terminal regions located within the prefrontal cortex (PFC). However, little evidence exists concerning the role of CRH_1 and CRH_2 receptors in mediating the effects of psychosocial stress. The present studies were conducted to expressly assess the contribution of CRH_1 and CRH_2 receptors at the DRN in the modulation of anxiety associated with stressors. To this end, we examined the effects of blocking CRH_1 or CRH_2 receptors prior to a social defeat stressor, both on anxiety-like behaviour in the elevated plus maze (EPM) and the accompanying forebrain variations of monoamine activity. Blocking CRH_1 or CRH_2 receptors reduced anxiety-like behaviour in the EPM in response to the stressor. Moreover, many of the actions of the social defeat stressor on central monoamine activity were attenuated by pre-treatment with the CRH receptor antagonists, depending on the brain region examined. These data indicate that antagonism of CRH_1 receptors or CRH_2 receptors at the dorsal raphe nucleus affect anxiety-related behaviours through variations of central monoamine functioning.

1. Introduction:

Stressful life events can adversely influence an individual's physical and mental well-being, including vulnerability to developing psychopathologies such as anxiety- and depressive-like disorders (Homberg et al., 2009). Serotonergic mechanisms, among other neurotransmitters, have been implicated in the symptomatology of various affective disorders, and targeting serotonin (5-HT) receptors (i.e. with selective serotonin reuptake inhibitors) may reduce anxiety- and depressive-like symptoms in both humans and rodents. However, stress-induced changes of other brain-derived hormones and peptides, including corticotropin-releasing hormone (CRH), have also been implicated in affective disorders. Although the interaction between CRH and 5-HT might be significant in mediating stress-related behavioural outcomes (Lukkes et al., 2009), limited data are available concerning their reciprocal actions in the prefrontal cortex (PFC) and hippocampus, both of which may be involved in anxiety and depression.

Stressors can promote or exacerbate many chronic illnesses and disorders (Bach et al., 2006; Caspi et al., 2003; Khasar et al., 2008; McEwen, 1998), especially if it is prolonged. For example, more than half of patients with major depressive disorder (MDD) show chronic activation of the HPA axis, and consistently elevated basal levels of cortisol (Arborelius et al., 1999; Checkley 1996; Holsboer, 2000, 2001; Kasckow et al., 2001). In line with this finding, those with MDD also tend to have a blunted adrenocorticotropin-releasing hormone (ACTH) response to intravenous injections of CRH (Gold et al., 1986; Holsboer et al., 1984). As well, several post-mortem studies assessing the brains of depressed patients have revealed important changes in the ratio of hippocampal mineralocorticoid and glucocorticoid receptor mRNA expression, the receptors responsible for cortisol secretion. Importantly, the receptor disturbances

were reversible by treatment with imipramine and desipramine, in-vivo (Lopez et al., 1998). Together, these data suggest a strong link between HPA axis and CRH dysfunction and mood regulation.

Corticotropin-releasing hormone (CRH) has marked effects on brain 5-HT activity, predominantly in limbic regions that mediate the response to stressors (Kirby et al., 2000; Lowry et al., 2000; Price et al., 1998, 2002). Conversely, brain serotonergic functioning may also reciprocally influence behaviour associated with changes of CRH (Hammack et al., 2002; Iwasaki-Sekino et al., 2009). Considerable evidence has illustrated that stressful events affect CRH activity not only at the periventricular nucleus of the hypothalamus (PVN), but also in the central amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) (Haugher et al., 2009; Koob & Heinrichs, 1999; Moore et al., 2000; Shekhar et al., 2005).

Stressful events are known to influence 5-HT activity in the PFC and hippocampus, reflecting increased activation of the dorsal raphe nucleus (DRN), a major site of 5-HT cell bodies which sends 5-HT projections to the forebrain. It is particularly significant from the present perspective that DRN neuronal activity is influenced by CRH (Thomas et al., 2003). The, CRH receptors are expressed uniquely in brain and are subdivided into CRH_1 and CRH_2 subtypes, located dissimilarly in the central nervous system (CNS). Whereas CRH_1 receptors are found throughout the CNS, particularly in limbic and forebrain regions, CRH_2 receptors are limited primarily to subcortical brain regions, including the lateral septum and the raphe nuclei. Uniquely, the dorsal raphe nucleus contains primarily CRH_2 receptors, which have been shown to mediate behavioural aspects of uncontrollable stressors (Hammack et al., 2002). Given the link between the DRN-mediated CRH actions and forebrain 5-HT variations, the present investigation assessed whether CRH infusion into the DRN would, in fact, promote anxiety-like

behaviour in mice, and whether these behavioural changes would be accompanied by monoaminergic variations within the PFC and hippocampus. As well, we examined whether blockade of CRH_1 or CRH_2 receptors via administration of the CRH_1 receptor antagonist (CP 154,526) or the CRH_2 receptor antagonist (astressin) would attenuate anxiety-like behaviours associated with a potent stressor comprising social defeat.

1.1. CRH and stress

Corticotropin-releasing hormone (CRH) is an amino acid peptide that has been shown to mediate endocrine (Vale et al., 1981), autonomic (Brown & Fisher, 1985), and behavioural (Koob et al., 1993) responses to stressors, and it plays a key role in adjusting the stress-activated hypothalamic-pituitary-adrenal (HPA) axis. Both CRH receptors and CRH actions have been linked to processes involved in mood regulation (De Souza et al., 1995; Gutman et al., 2000; Holsboer, 1999; Keck et al., 2001) as well as various immune and cognitive processes (De Souza et al., 1995; Dieterich et al., 1997). During and after a stressful event, CRH is released from the paraventricular nucleus (PVN) of the hypothalamus to stimulate the anterior pituitary gland, releasing adrenocorticotropin-releasing hormone (ACTH). This, in turn, stimulates the adrenal cortex, causing secretion of glucocorticoids (cortisol in humans or corticosterone in rodents) into the blood stream. These specific CRH-related processes are critical in initiating the classic neuroendocrine response to stress that includes the secretion of cortisol from the adrenal gland (Kageyama & Suda, 2009).

Central administration of CRH tends to produce effects similar to those produced by stressors, including autonomic and behavioural effects such as increased heart rate and blood pressure (Fisher et al., 1982), as well as fluctuations in gastrointestinal functioning (Tache et al., 1993). Exogenous CRH administration has also been shown to enhance fear responses (Butler et

al., 1990), provoke reductions in exploratory behaviours in novel environments (e.g. open field or elevated plus maze) (Sutton et al., 1982; Berridge & Dunn, 1989), and cause disturbances in sleep (Sherman & Kalin, 1988) and food intake (Morley & Levine, 1982). Since these anxiety-like characteristics can be induced by exogenous CRH and by stressors, coupled with the fact that these symptoms may be attenuated by administration of CRH receptor antagonists, the hypothesis was formed that CRH receptor antagonists could be therapeutic in illnesses such as major depressive disorder. In fact, a number of animal models involving CRH have since been utilized, including genetic animal models comprising CRH receptor knockout mice, and pharmacological studies examining the effects of selective CRH receptor antagonism. Together, these approaches supported a link between hyperactive CRH activity and depressive- and anxiety-like behaviours. Although the CRH_2 receptor has been identified as a potential target for the treatment of anxiety and/or depression (Bale et al., 2002), much more is known concerning the anxiolytic effects of blocking the CRH_1 receptor.

As mentioned earlier, administration of exogenous CRH consistently produced anxiogenic effects on behaviour in rodents, primarily by CRH_1 but not CRH_2 receptor activation (Muller et al., 2003, Nguyen et al., 2006; Sahuque et al., 2006). However, at higher doses, CRH appears to induce a shift from CRH_1 activation toward CRH_2 activation, and it is conceivable that under these conditions CRH_2 might play a more prominent role in anxiety. Potent stressors, including chronic and uncontrollable types, likewise result in CRH_2 receptor activation, and it has thus been suggested that the type of CRH receptor activated likely depends on specific stressor characteristics. Thus, the stress-induced shift from CRH_1 to CRH_2 activation that has been shown to occur after re-exposure to stressors may be important with respect to affective disorders.

Hammack et al. (2002) demonstrated that CRH_2 receptors in the DRN mediate the behavioural effects of uncontrollable stressors; a finding that was replicated in the lateral septum and reversed by pretreatment of selective CRH_2 antagonists (Bakshi et al., 2002). It has also been suggested that CRH_2 receptors might influence stress-related behaviour through interactions with other neurotransmitters, independent of HPA axis activity, but the precise function of these receptors remains somewhat controversial. Although it has been demonstrated that both receptors can reduce anxiety-like behaviour in rodents (Takahashi et al., 2001), activation of CRH_1 and CRH_2 receptors was also reported to provoke opposing behavioural outcomes (Ji & Neugebauer, 2007; Zhao et al., 2007), depending on the brain region in which the drug is infused. Thus, limbic and other extrahypothalamic regions containing CRH receptors may be a target of particular importance in the treatment of stress-related disorders, as in the case of administering CRH_1 and CRH_2 receptor antagonists (Kehne et al., 2002).

1.2. CRH and CRH receptors: Novel targets for the treatment of affective disorders

As previously mentioned, corticotropin-releasing hormone (CRH) receptors are widely distributed in the central nervous system (CNS), projecting from hypothalamic and extrahypothalamic (e.g. CeA and BNST) sites to areas including the cerebral cortex (Kehne et al., 2002). Indeed, CRH_1 receptors are expressed similarly between species, and in high abundance in the pituitary, where they modulate HPA axis functioning. They are also found in the cerebral and cerebellar cortices, limbic system, olfactory bulb, thalamus, and brainstem (Kehne et al, 2002). Conversely, CRH_2 receptors are localized dissimilarly between species and are restricted more to the periphery and brain regions such as the lateral septum (LS), ventromedial hypothalamus, central amygdala (CeA) and raphe nuclei (Chalmers et al., 1995; Chen et al., 2000; Van Pett et al., 2000).

In characterizing the functional properties of CRH receptors, a wide variety of pharmacological treatments have been developed and utilized with animal models. The most common agents have been peptidergic antagonists with varying affinities for CRH_1 and CRH_2 receptors, such as alpha-helical CRH, and D-Phe CRH. However, their potency and/or receptor selectivity has limited their use experimentally. As such, extensive efforts continue to be devoted to uncovering potent, non-peptidergic, small-molecule CRH antagonists for the CRH_1 receptor, which so far, appears more promising than the CRH_2 receptor as a treatment for depression and/or anxiety.

Studies using genetically engineered mice revealed that over-expression of CRH produced anxiogenic behaviours in an elevated plus maze. Further, anxious behaviour exhibited in the elevated plus maze following CRH administration could be reversed by the non-selective CRH receptor antagonist, alpha-helical CRH, but not by adrenalectomy. These results are consistent with the premise that central CRH receptor mediation can occur independently of the HPA axis (Stenzel-Poore et al., 1994), illustrating the importance of extrahypothalamic regions in mediating CRH actions. Although CRH receptor activity in the PVN and in other limbic regions of the brain have been linked to 5-HT functioning as well as in various affective disorders (i.e. depression, anxiety), the DRN may be especially important in regulating 5-HT activity elicited by CRH. By elucidating the reciprocal CRH-5-HT connections between the DRN and the forebrain, it might be possible to target CRH receptors in order to reduce symptoms of depression and/or anxiety.

Following inactivation of the CRH_1 receptor with an antisense oligonucleotide, specific CeA involvement of the CRH_1 receptor in anxiolytic-like outcomes was apparent (Liebsch et al., 1995). Further, knockout mice lacking CRH_1 receptors elicited reduced anxiety on the elevated

plus maze, and also decreased ACTH and corticosterone responses to restraint stressors (Smith et al., 1998). Moreover, using CRH_1 and CRH_2 receptor antisense oligonucleotides, Heinrichs et al. (1997) demonstrated that the anxiogenic actions of CRH were mediated by CRH_1 but not CRH_2 receptors. These anxiogenic effects on behaviour were hypothesized to occur through actions of CRH on the locus coeruleus (LC) in the noradrenergic system.

1.3. The Dorsal Raphe Nucleus (DRN)

There has been increased interest in the organization of the DRN, driven by its role in regulating physiological systems and behavioural functions through 5-HT functioning (de Almeida et al., 2005; Jacobs et al., 1978; Layer et al., 1992; Lucki, 1998; Maes and Meltzer, 1995; McGuirk et al., 1992; Simansky, 1996; Vertes, 1988). Due to its involvement in mediating stress-induced behaviour, along with the fact that CRH - 5-HT interact at the DRN, dysregulation of the DRN has been linked to numerous stress-related disorders (Carrasco & Van de Kar, 2003; Graeff et al., 1996). Indeed, several studies suggested that interactions between CRH and serotonergic systems may be important in regulating anxiety-behaviour in rats (Lowry et al., 2005; Maier & Watkins, 2005) and in anxiety and depressive disorders in humans (Arborelius et al, 1999; Austin et al., 2003).

Although the DRN is the largest 5-HT containing nucleus in the brainstem, a large portion of DRN cell bodies are non-serotonergic (Molliver et al., 1987; Moore et al., 1981). Clusters of 5-HT neurons are co-localized among other non-5-HT neurons (Molliver, 1987; Moore, 1981), suggesting that other neurotransmitter and neuropeptides likely influence the DRN and the activation of DRN 5-HT neurons. Among the non-5-HT neurons identified within the DRN are neurons expressing CRH (Commons et al., 2003), among others. Serotonergic neurons localized on the DRN are also known to be under tonic inhibition by somatodendritic 5-

$HT1_A$ neurons and GABAergic interneurons (Tao et al., 1996), and this inhibition is controlled by $GABA_A$ receptors (Celada et al., 2001).

As stated previously, the DRN uniquely contains more CRH_2 receptors than CRH_1 receptors, and both receptors have different mechanisms of activation. Although activation of CRH_1 and CRH_2 receptors have been shown to elicit opposing behavioural responses to stressors (Ji & Neugebauer, 2007; Zhao et al., 2007), stimulation of CRH_1 receptors on the DRN causes an inhibition of DRN-5-HT activity, whereas stimulation of DRN-situated CRH_2 receptors stimulates DRN-5-HT neurons and increases 5-HT activity. Much like the effects produced by stressors, low doses of CRH tend to activate primarily CRH_1 receptors, with higher doses activating primarily CRH_2 receptors. Again, this shift from stimulation of CRH_1 to that of CRH_2 receptors may be integral in shaping the behavioural outcome and shifting the type of response from active to passive.

1.4. The link between the DRN and the PFC: CRH-5-HT interactions

The prefrontal cortex (PFC) processes highly complex information and is thought to be directly involved in assessing the controllability of stressors. Further, the PFC is involved in the assessment of the salience of stressors and the subsequent formation of appraisals, whereby stressor information, such as the controllability and potency of the stressor, is evaluated and processed (Amat et al., 2005). During the appraisal of stressful events, complex information from the PFC is processed and relayed through limbic and infra-limbic pathways back to the DRN, leading to CRH and 5-HT cell activation (Hammack et al., 2003; Jankowski et al., 2004). Stimulation of these receptors then provokes specific behavioural responses through 5-HT afferents to the forebrain. The vast majority of 5-HT innervation to the mPFC originates in the dorsal raphe nucleus (Bjorklund et al., 1971; Descarries et al., 1975; Li et al., 1989; Mantz et al.,

1990; North and Uchimura, 1989). The evidence linking DRN collateralized projections to the medial prefrontal cortex (mPFC) and lateral prefrontal cortex (lPFC) is, in fact, fairly limited. However, it has been shown that the lPFC and mPFC both provide projections back to the DRN (Goncalves et al., 2009; Roberts, 2011), and the reciprocal connectivity between the DRN and these forebrain structures have been hypothesized to be fundamental in shaping the behavioural response to stressors. Further, it was reported that these connections may be involved in directing motivated behaviours, including impulsivity, attention to various stimuli, and integrative learning (Maier et al., 2006; Perry et al., 2011; Roberts, 2011). In line with results reported by Goncalves et al. (2009) and Roberts et al. (2011), Lowery (2002) had previously proposed that 5-HT neurons project from the caudal region of the DRN to limbic and cortical structures from a mesocorticolimbic 5-HT system involved in mediating anxiety. Furthermore, Gardner et al. (2005) reported that a single exposure to a social defeat stressor selectively activates DRN 5-HT neurons. In the main, activation of CRH_2 receptors by CRH or CRH-related ligands is thought to be at the core of this process, and it is possible that CRH_2 receptors may be more critical than CRH_1 receptors under conditions of stress comprising social inconsistency and/or defeat.

The goals of the present studies were to assess the contribution of the CRH-5-HT connection between the DRN and the PFC, and particularly to examine how changes in CRH affect behaviour associated with a potent stressful event. It was hypothesized that exogenous CRH administration would increase 5-HT turnover in the PFC and hippocampus, causing anxiogenic behavioural effects (i.e. reducing exploration behaviour), whereas administration of the CRH_1 or CRH_2 receptor antagonists would have anxiolytic effects on behaviour, leading to a reduction of 5-HT turnover in the PFC and hippocampus.

In the present investigation we used a brief, but potent uncontrollable stressor in which a social encounter with a retired breeder mouse occurred. This experience generally leads to social defeat and evokes a pronounced stress response accompanied by changes in neurotransmission. The choice to use a social defeat stressor was based on its translational value to social stress in humans and its naturalistic characteristics (Bjorkqvist, 2001). We examined whether CRH_1 or CRH_2 receptor antagonism would block social defeat-induced anxiety-like behaviour on the open field and elevated plus maze. In this instance, we assessed the effects elicited by pre-treatment with a selective non-peptide CRH_1 receptor antagonist (CP 154,526) or a selective CRH_2 receptor antagonist (astressin). It was also of interest to determine whether CRH_1 or CRH_2 receptor antagonism would produce changes in central monoamines in forebrain and limbic regions following social defeat stress. The choice of astressin was based on its selectivity for the CRH_2 receptor, and the use of CP 154,526 was based on its receptor selectivity and its ability to readily cross the blood brain barrier (Schulz et al., 1996).

2. Methods:

2.1. Animals

Male CD-1 mice were obtained from Charles River (Quebec) at 50–60 days of age and were acclimatized to the laboratory for approximately 7 days before serving as experimental subjects. Mice were single-housed per cage from arrival until time of sacrifice. The vivarium was maintained on a 12-h light/dark cycle in a temperature-controlled (21 °C) room with food and water freely available. All experiments complied with the guidelines set by the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee.

2.2. Surgical procedure: Cannulations

Mice were anesthetized using isoflurane and stereotaxic surgery (David Kopf Instruments Model 940) was performed to install a cannula into the dorsal raphe nucleus. A guide cannula (Plastics One In) was situated according to ref. 67 at lateral = 2.02 mm, dorsoventral = 3.40 mm and anteroposterior = -4.36 mm. Approximately 1 week after behavioural testing, mice were sacrificed by perfusion with 4% paraformaldehyde. Brains were subsequently sectioned at 14 μm and stained with Cresyl violet for probe placement verification. Only the data from mice with correct probe placements were included in the statistical analysis.

2.3. Drug treatments

Unless otherwise specified, all experiments used a drug diffusion that was permitted for 5 minutes while mice were resting, and mice were then either placed in another dominant male's cage for a 5 minute social defeat session, or were monitored for anxiety-related behaviours (see Behavioural testing section). Then, each mouse was put through behavioural testing in the open field test for 5 minutes and an elevated plus maze for 5 minutes almost immediately after the 5 minute rest that followed drug infusion. Behavioural testing was conducted 5 minutes after drug infusion with 1 minute between open field and elevated plus maze sessions.

In Experiment 1, mice were infused with one of three doses of CRH (0.375, 0.750, and 1.5ug) or vehicle (saline) into the DRN. This occurred 1 week after surgical recovery, over a 5-minute period, through an internal cannula situated 0.3 mm below the guide cannula, while mice were briefly anesthetized by isoflurane. We assessed the effects of the three CRH doses on anxiety-like behaviour in the OF and EPM. These drug doses were piloted in order to find the

most appropriate dose that affected anxiety-related behaviours while minimizing disruption of locomotor activity.

In Experiment 2, we assessed the effects of the moderate dose of CRH (0.750ug) administered into the DRN, on MHPG (metabolite of Norepinephrine), on 5-HIAA (metabolite of 5-HT), and on DOPAC (metabolite of Dopamine) in the PFC and hippocampus. The exact procedure from Experiment 1 was repeated except mice were sacrificed and brains were harvested for HPLC. This data provided the accompanying central monoaminergic outcomes pertaining to the behaviour that was examined in Experiment 1.

In Experiment 3, we assessed the effects of social defeat on anxiety-related behaviours following pre-administration of 0.2ug of astressin into the DRN, a selective CRH_2 receptor antagonist. As in the previous experiments, administration of the drug was given prior to the open field and elevated plus maze, and mice either received a 5 minute social defeat stressor or were not stressed (control group).

In Experiment 4, we assessed the effects that social defeat had on 5-HT and 5-HIAA concentrations in the PFC and hippocampus, also following the administration of 0.2ug of astressin. The exact drug infusion and stressor procedure was repeated from Experiment 3 except mice were sacrificed 5 minutes after the stressor, and brains were harvested for HPLC. This data provided the accompanying central variations of 5-HIAA and 5-HT pertaining to the behaviour that was examined in Experiment 3.

In Experiment 5, we assessed whether administration of a CRH_1 receptor antagonist, CP 154,526 (10mg/kg, i.p), before the social defeat stressor, would attenuate stress-induced

anxiogenic behaviour in the open field and elevated plus maze. Mice were pre-treated with the drug or vehicle (saline suspended in 0.5% Carboxymethylcellulose (CMC)), exposed to the 5 minute social defeat stressor, and then tested in each task for 5 minutes. In this experiment, CP 154,526 was injected 30 minutes prior to the 5 minute social defeat stressor, and the time between the end of the stressor and the behavioural paradigms were reduced from 5 minutes to 3 minutes.

In Experiment 6, we assessed the stress-induced variations of central monoamine activity following administration of the same CRH_1 receptor antagonist used in Experiment 5. The exact procedure from Experiment 5 was repeated except mice were sacrificed 3 minutes after the social defeat stressor instead of behavioural testing. Brains were harvested for monoamine evaluation by HPLC, based on tissue from the PFC, hippocampus, PVN, and CeA. The drug dose used in Experiments 5 and 6 (10mg/kg, i.p.) was based on the fact that CP 154,526 readily crosses the blood-brain barrier, peaks in concentration in roughly 30 minutes, and has been used effectively with mice in various stress-related behavioural tasks (see Shulz, 2002; Lowery et al., 2008).

2.4. Behavioural testing

In an initial test, mice were placed in a 45 × 45 cm open field, with an inner square of 21 × 24 × 24 cm, for a 5 minute period, during which the time to enter the center area and the total time spent in the center portion of the arena was recorded and videotaped. The elevated-plus maze test was then conducted 1 minute after the open field assessment. The elevated plus maze had two arms enclosed by 21-cm-high walls; whereas the remaining two arms were open (24.8 × 7.7 cm). The maze was situated in a dimly lit room, such that the closed arms were darkened, whereas open arms were somewhat illuminated. Mice (n= 8-10 per group) were individually

placed in one of the enclosed arms of a plus-maze and the behaviour of the mice was recorded over a 5 minute period by a ceiling-mounted video camera. The amount of time spent in each of the arms and the number of arm entries (an arm entry was defined as all four of the paws being placed in an arm of the plus maze) were recorded. All behavioural experiments were blinded, data were obtained from videotapes, and the researcher was blind as to the treatments the mice had received.

2.5. Blood collection and brain removal

Between 0800 and 1000 hr mice were sacrificed by rapid decapitation. Trunk blood was collected in tubes containing 10 μg of EDTA, centrifuged for 8 min at 3600 RPM, and the plasma stored at -80 °C for subsequent corticosterone determination. Brains were rapidly removed and placed on a stainless steel brain matrix (2.5 × 3.75 × 2.0 cm) positioned on a block of ice. The matrix comprised a series of stainless steel plates that had a series of slots spaced 500 μm apart that guided razor blades to provide coronal brain sections. Once the brains were sliced, tissue punches from the PFC and hippocampus were collected by micropunch using hollow 20 gauge needles with a bevelled tip following the mouse atlas of Franklin and Paxinos (1997). The collection of these punches took no longer than 2 minutes following the decapitation of the animal. Tissue punches were stored at -80°C for subsequent determination of cytokine or 5-HT receptor mRNA expression. For samples that were to be used for monoamine determinations, the tissue punches were placed in 0.3 Monochloroacetic acid containing 10% methanol and internal standards, and then stored at –80°C.

2.6. High Performance Liquid Chromatography (HPLC) assay

The levels of DA, NE and 5-HT, and their metabolites, DOPAC, MHPG and 5-HIAA, were determined by HPLC. Tissue punches were sonicated in a homogenizing solution comprising 14.17 g monochloroacetic acid, 0.0186 g disodium ethylenediamine tetraacetate (EDTA), 5.0 ml methanol and 500 ml H_2O. Following centrifugation, the supernatants were used for the HPLC analysis. Using an Agilent (Mississauga, Ontario) pump, guard column, radial compression column (5m, C18 reverse phase, 8mm x 10cm), and coulometric electrochemical detector (ESA Model 5100,A), 40 µl of the supernatant was passed through the system at a flow rate of 1.5 ml/min (1400-1600 PSI). Each liter of mobile phase consisted of sodium dihydrogen phosphate (90mM), 1-octase sulfonic acid (sodium sal) (1.7 mM), EDTA (50mM), citric acid (50 mM), potassium chloride (5 mM) and 10% acetonitrile. The mobile phase had been filtered (0.22 mm filter paper) and degassed. The area and height of the peaks were determined using an Agilent integrator. Protein content of each sample was determined using bicinchoninic acid with a protein analysis kit (Pierce Scientific, Brockville, Ontario) and a Fluorostar colorimeter (BMG, Durham, NC). The lower limit of detection for the monoamines and metabolites was approximately 1.0 ρg.

2.7. Data analysis

The mean and the s.e.m. were expressed for values obtained from the number of separate experiments indicated. Dose response data were analyzed using GraphPad Prism (GraphPad Software). Statistical significance was determined by analysis of variance. Data were analyzed by either a single factor (drug infusion) or two factor (drug infusion x stressor) analysis of variance, as appropriate, independently for each of the outcome measures. Follow-up tests were

3. Results

3.1. Experiment 1: The effects of intra-DRN administration of CRH on anxiety-like behaviour in the OF and EPM

The CD-1 mice tested in the elevated plus maze demonstrated anxiety-like behaviours that varied as a dose-dependent function of CRH treatment, with respect to the latency to enter the open arms, $F(3,57) = 4.05$, $p < .05$, and the time spent there, $F(3,57) = 3.58$, $p < .05$. Follow-up comparisons of the simple effects of the three CRH doses indicated that all three doses (CRH 1.5ug, CRH 0.750ug, and CRH 0.375ug) significantly increased the latency to enter the open arms compared to vehicle-treated mice ($p < .05$, $p < .01$, and $p < .05$ respectively). Although the moderate dose (CRH 0.750ug) appeared to provoke the largest effect on latency, the three doses did not, in fact, differ significantly from each other in their anxiogenic effects on behaviour, nor did they provoke any differences, compared to the vehicle group, in the time spent in the closed arms of the EPM, $F(3,57) = 1.05$, $p = .38$, (see Figure 1 below). However, compared to the vehicle group, the CRH-treated mice spent significantly less time in the open arms of the EPM, and these effects were dependent on the CRH dose. Although the highest dose (CRH 1.5ug) did not quite reach statistical significance compared to vehicle-treated mice, $p = .054$, the moderate and low doses did ($p < .05$ and $p < .01$ respectively). In the open field, overall, the latency to enter the inner square did not vary as a function of the CRH treatment. However, based on a priori hypotheses, follow up tests were performed. These revealed that only the middle dose (CRH 0.750ug) provoked a significant increase in the latency to enter compared to the vehicle-treated mice ($p < .05$) (See Figure 2 below).

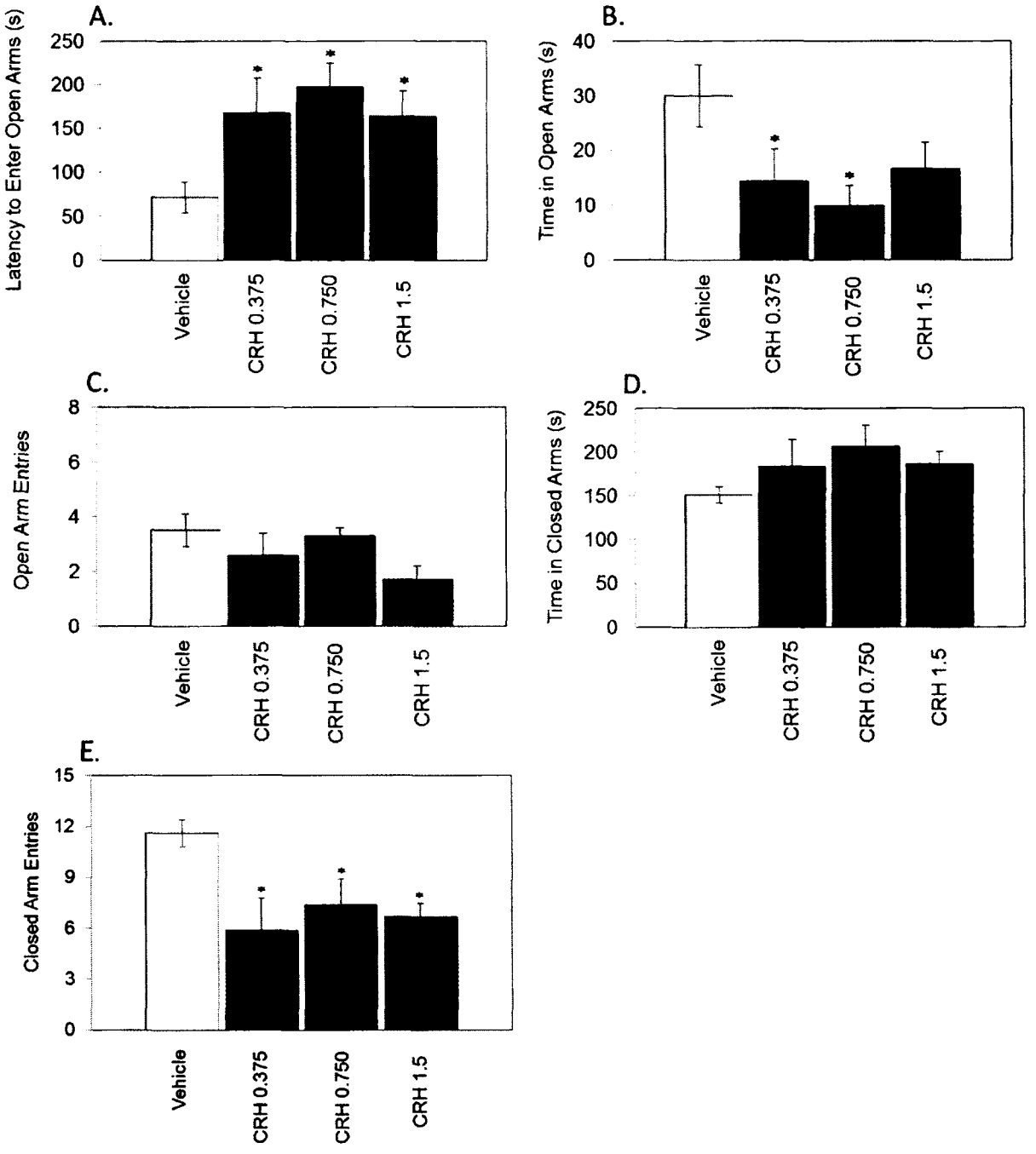

Figure 1. Mean + SEM plus maze behavioural changes as a function of the CRH treatment (dosage in μg) that mice received. All 3 CRH doses increased the latency to enter the opens arms (see Panel A), reduced the number of open arm entries (see Panel B), and decreased the amount of time spent in the open arms (see Panel C). There were no significant differences between groups with respect to the time spent in the closed arms of the EPM (see panel D). However, the CRH treatments did provoke reductions in the number of entries into the closed arms, compared to the vehicle-treated mice (see Panel E).

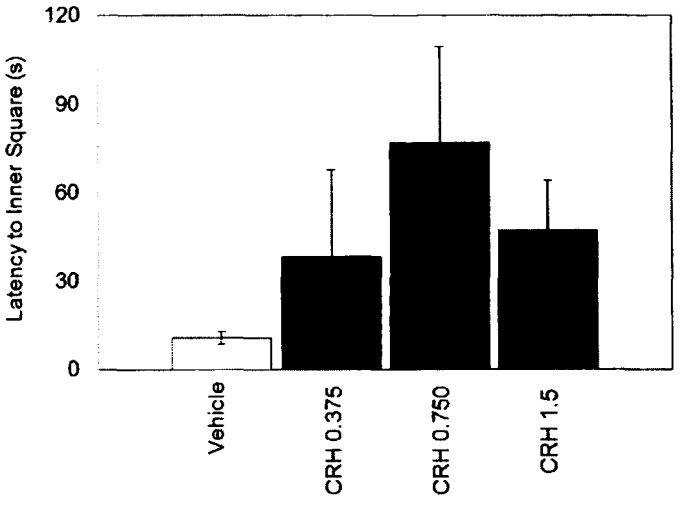

Figure 2. Mean + SEM open field behavioural changes as a function of the CRH treatment (dosage in ug) that mice received. Only the middle dose (CRH 0.750ug) provoked a significant increase in the latency to enter the inner square compared to the vehicle-treated mice.

3.2. Experiment 2: The effects of intra-DRN administration of CRH on central monoamine metabolites in the PFC and hippocampus

The intra-DRN administration of the moderate dose of CRH (0.750ug) provoked significant increases in the accumulation of DOPAC, $F(1,47) = 7.38, p < .01$. Further, 5-HIAA concentrations in the PFC also varied as a function of the CRH-treatment, $F(1,47) = 8.65, p < .01$, but not for MHPG, $F(1,47) = .42, p = .52$. Similar to the variations in the PFC, 5-HIAA accumulation in the hippocampus also varied as a function of the drug treatment, $F(1,47) = 14.98, p < .001$. Although the CRH treatment provoked a large increase in 5-HIAA concentrations, MHPG concentrations were unaffected, $F(1,47) = .32, p = .57$ (see Figure 3 below).

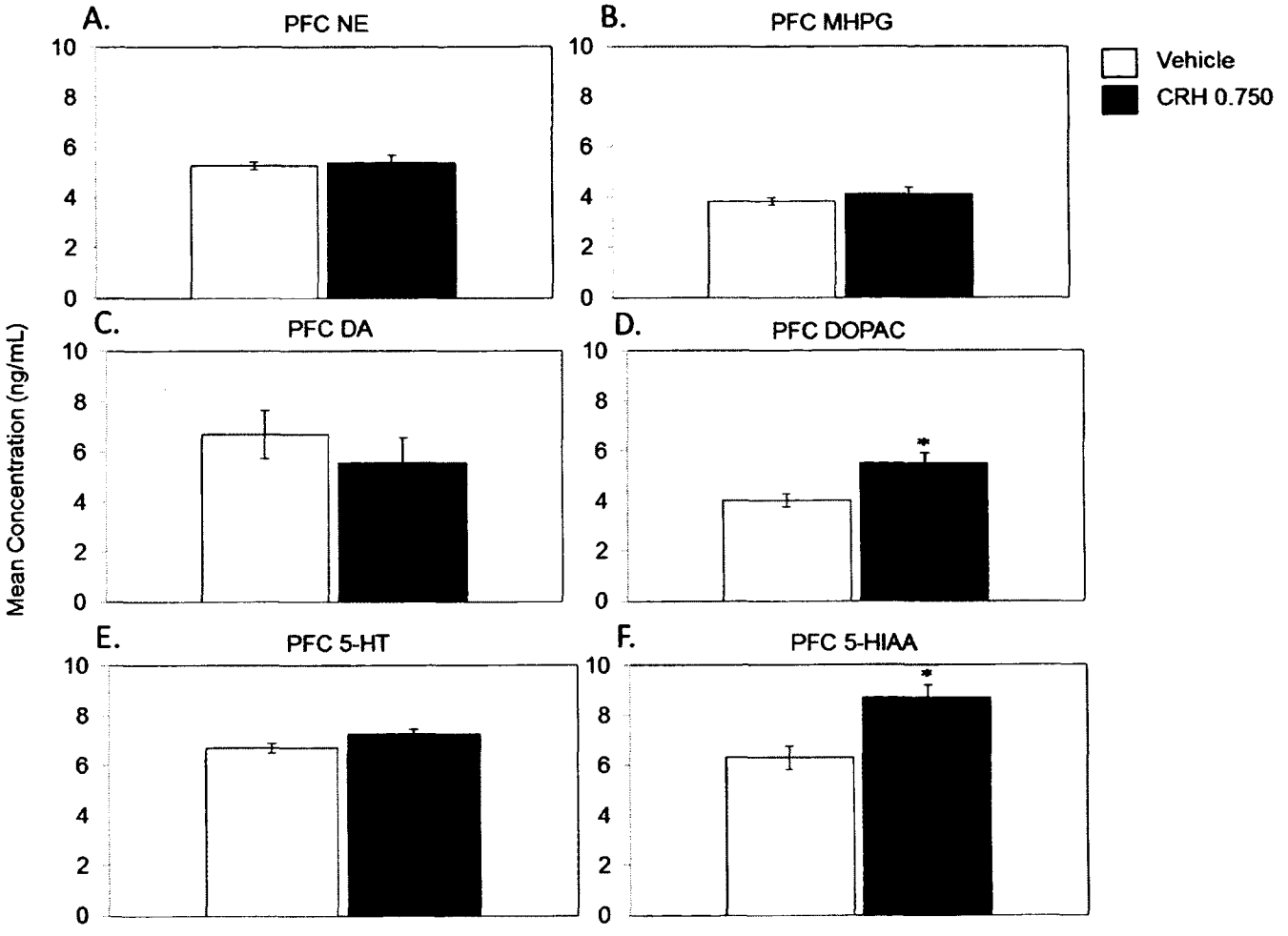

Figure 3. Mean + SEM monoamine changes in prefrontal cortex as a function of the CRH treatment (dosage in μg) that mice received. The middle dose (0.750μg CRH) provoked significant variations in DOPAC and 5-HIAA concentrations in the PFC (see Panel D and F, respectively).

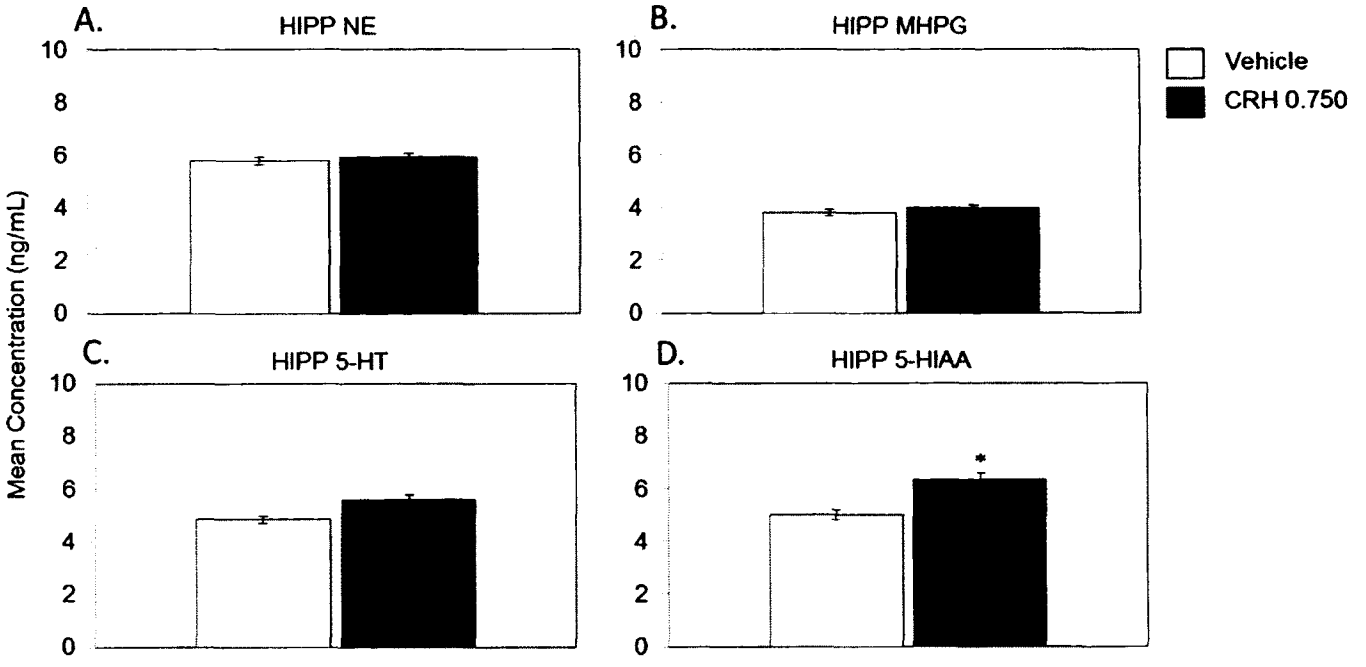

Figure 4. Mean + SEM monoamine changes in hippocampus as a function of the CRH treatment (dosage in ug) that mice received. The middle dose (0.750ug CRH) provoked an increase in 5-HIAA in the hippocampus, similar to that produced in the PFC (see Figure 3 above).

3.3. Experiment 3: The effects of CRH_2 receptor antagonism in the DRN on social defeat-induced anxiety-like behaviour in the EPM

The CD-1 mice tested in the elevated plus maze demonstrated latencies to enter the open arms that varied as a function of the Drug x Stressor interaction, $F(1,34) = 25.06$, $p < .0001$, with no effect on the number of entries or time spent there. In the non-stress group, the vehicle-treated mice were slow to enter the open arms of the EPM, and the CRH receptor antagonist reduced the time in half. However, under conditions of stress, the Drug x Stressor interaction produced the exact opposite result, potentiating the anxiety-like response (see Figure 5). There were no significant differences between groups with respect to the time spent in the closed arms of the EPM, $F(1,34) = .005$, $p = .95$ (see Figure 5 below).

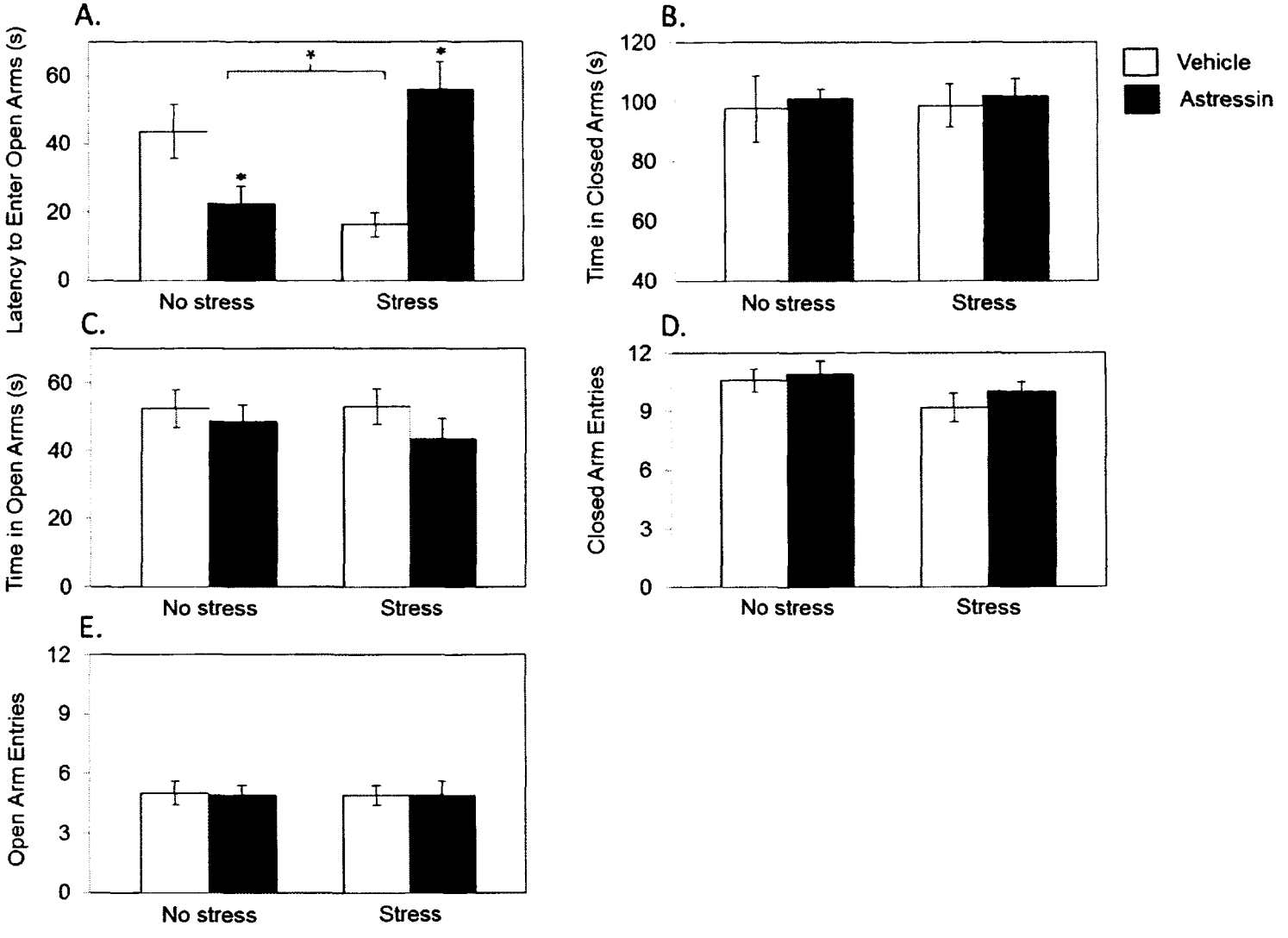

Figure 5. Mean + SEM plus maze behavioural changes as a function of the astressin treatment (dosage in μg) that mice received. Blocking CRH_2 receptors in the DRN (astressin, 0.2μg) prior to the social defeat stressor reduced the latency to enter to open arms of the EPM among the non-stressed mice, but this effect was the opposite among those in the stress group (see Panel A). The astressin treatment did not produce any other notable changes in anxiety-related behaviour in this regard.

3.4. Experiment 4: The effects of CRH_2 receptor antagonism in the DRN on social defeat-induced monoaminergic variations in the PFC

In the PFC, NE concentrations varied as a function of drug treatment, $F(1,42) = 4.6$, $p < .05$, reducing levels among only the non-stressed mice. Further, PFC MHPG accumulation varied as a function of the Drug x Stressor interaction, $F(1,42) = 4.5$, $p < .05$. Based on a priori hypotheses, follow-up comparisons of the simple effects of the vehicle and astressin treatments were performed. The astressin-treated mice demonstrated significantly lower levels of NE than the vehicle-treated mice, but this effect was only apparent in the non-stress group, $p < .05$. Further, the vehicle-treated mice demonstrated increased MHPG accumulation compared to those in the non-stress group, and this effect was modestly attenuated by pre-treatment with the CRH_2 receptor antagonist (See Figure 6).

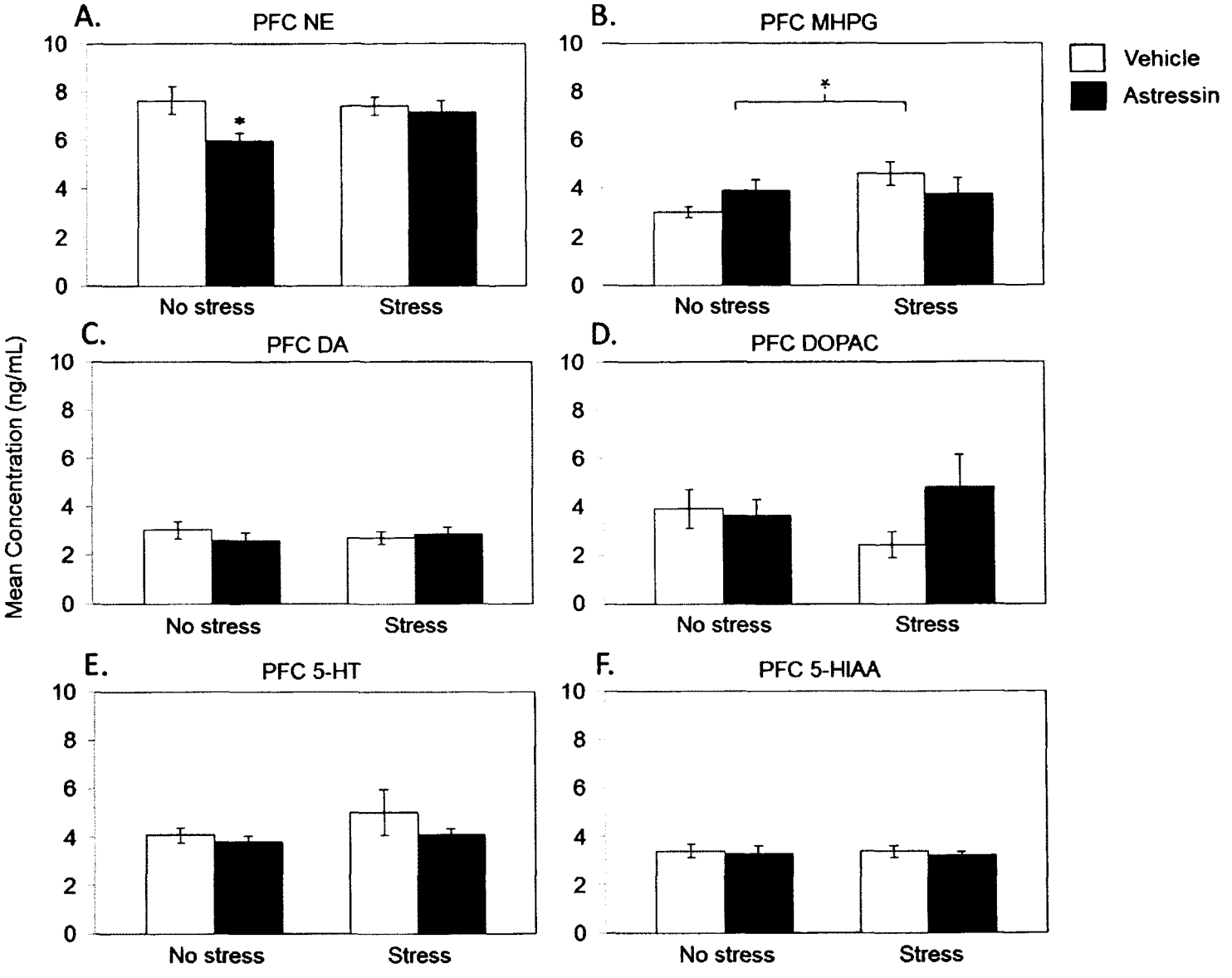

Figure 6. Mean + SEM monoamine variations in the prefrontal cortex as a function of the astressin treatment (dosage in μg) that mice received. There was a significant drug effect on NE levels in the PFC (see Panel A) and a significant Drug x Stressor interaction on MHPG concentrations within the PFC (see panel B).

3.5. Experiment 5: The effects of CRH_1 receptor antagonism on social defeat-induced anxiety-like behaviour in the EPM

Pre-administration of CP 154,526 (10mg/kg, i.p), 30 minutes prior to the social defeat stressor, significantly decreased the latency to enter the open arms of the EPM compared to the vehicle-treated mice, $F(1,39) = 34.86$, $p < .0001$, increased the total time spent there, $F(1,39) = 4.93$, $p < .05$, and increased the number of open arm entries, $F(1,39) = 13.59$, $p < .001$. Importantly, the vehicle- and drug groups did not differ with respect to the number of closed arm entries in the elevated plus maze, $F(1,39) = .18$, $p = .68$, implying no confounding drug effects on motor functioning. Although the main effect of the stressor on anxiety-like behaviour did not quite reach statistical significance, there was a significant Drug x Stressor interaction present, with respect to the mean latency to enter the open arms of the EPM, $F(1,39) = 4.4$, $p < .05$. The vehicle-treated mice in the stress group demonstrated shorter latencies to enter the open arms compared to mice in the non-stress group. However, this interaction is speculated to represent stress-induced impulsivity, provoked by the bullying manipulation, which has been a common finding in many of our experiments (see Figure 7 below).

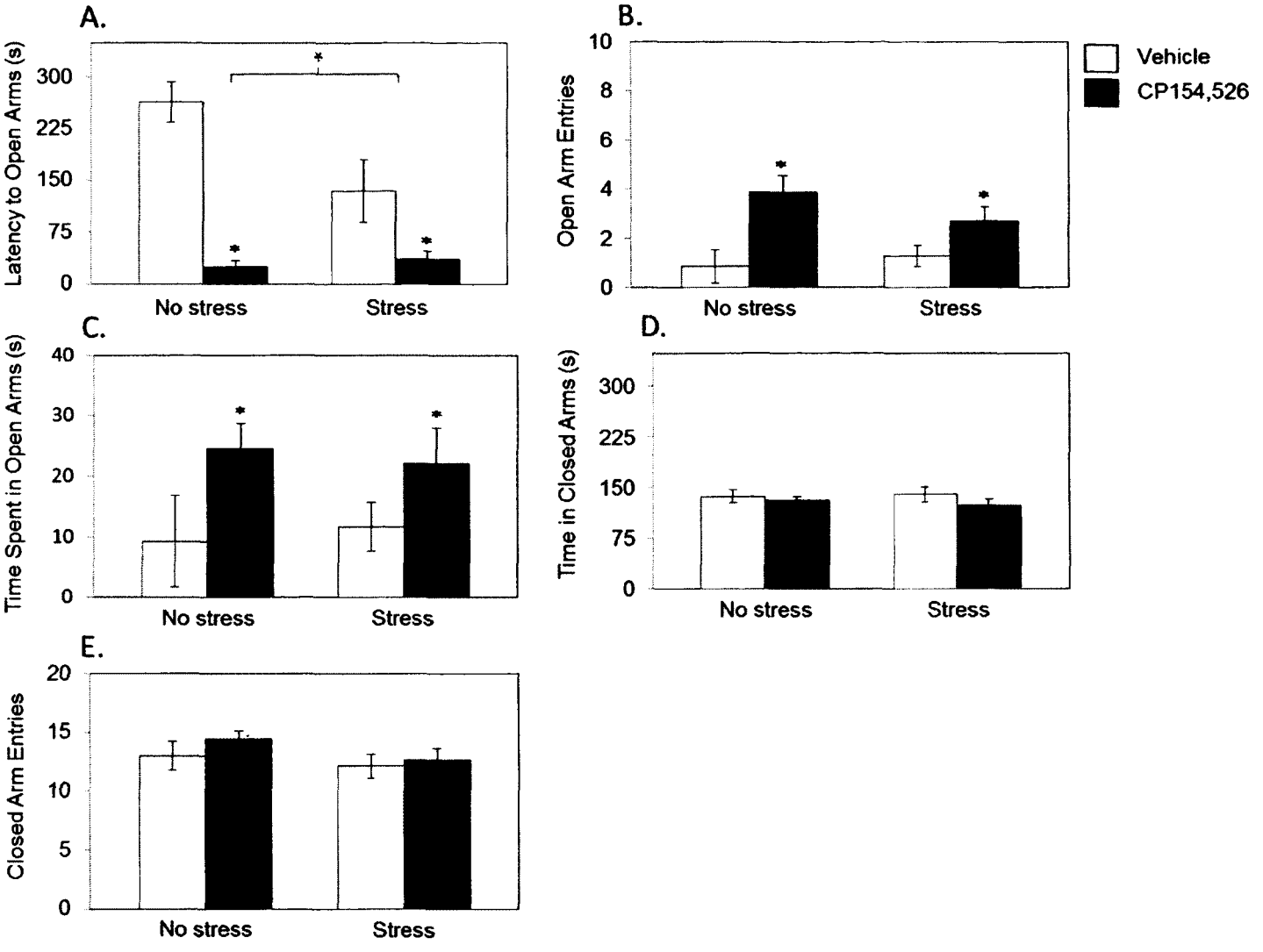

Figure 7. Mean + SEM plus maze behavioural changes as a function of the CP 154,526 treatment (dosage in mg/kg) that mice received. Blocking CRH_1 receptors with CP 154,526 (10mg/kg, i.p.) prior to social defeat exposure significantly reduced the latency to enter the open arms of the EPM (see panel A), increased the number of open arm entries (see panel B), and increased the time spent there (see Panel C). There were no significant differences between groups in the time spent in the closed arms or the number of entries there (see Panel D and E, respectively.)

3.6. Experiment 6: The effects of CRH_1 receptor antagonism on central monoamine variations in the PFC, CeA, hippocampus, and PVN

In the PFC, MHPG concentrations varied as a function of the stressor, $F(1,26) = 13.52$, $p < .005$, and as a function of the drug treatment, $F(1,26) = 6.01$, $p < .05$. Follow-up tests revealed a notable increase of MHPG concentrations among the drug-treated mice. Although the interaction was not significant, the effect of the drug treatment was most apparent in the stressed group relative to non-stressed mice (see Figure 8 below). In contrast to the metabolite change, NE itself was not affected by either the stressor or the CRH antagonist. The bullying manipulation elicited a slight increase of DOPAC accumulation, $F(1,26) = 3.68$, $p = .06$, whereas the level of DA was not altered. Finally, 5-HT levels were moderately reduced by the CRH antagonist, $F(1,26) = 4.01$, $p < .05$, but not by the stressor treatment. The concentrations of 5-HIAA were unaffected by either treatment (see Figure 8 below).

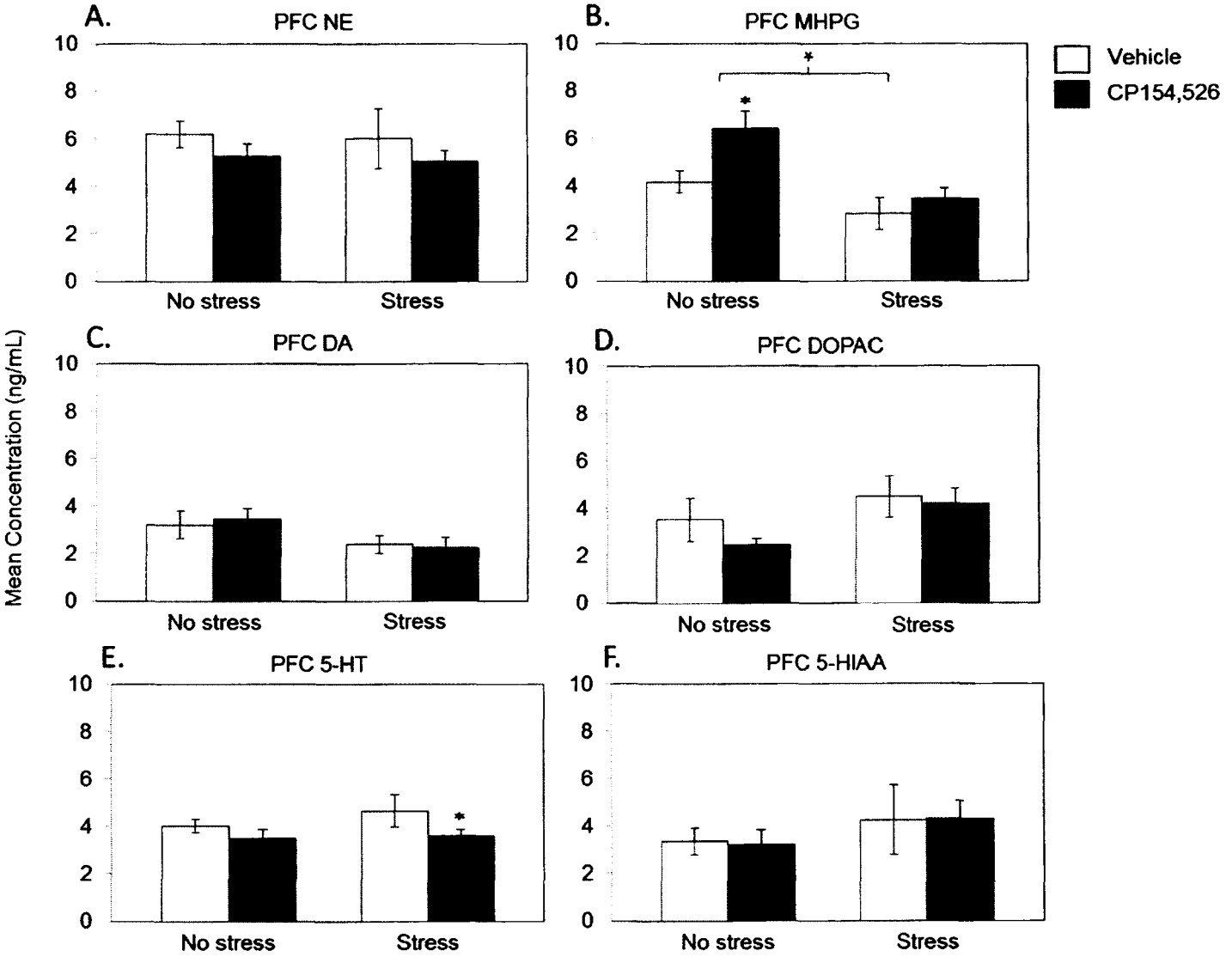

Figure 8. Mean + SEM monoamine variations in the prefrontal cortex as a function of the CP 154,526 treatment (10 mg/kg, i.p.) that mice received. There were significant drug and stressor effects on MHPG accumulation within the PFC (see Panel B), as well as a modest reduction of DOPAC accumulation in the non-stress group due to the drug treatment (see panel D), and also a significant drug effect on 5-HT (see Panel E).

In the PVN, there was a modest variation of MHPG accumulation as a function of the stressor, $F(1,22) = 3.84$, $p = .06$, with no drug effect or Drug x Stressor interaction. Follow-up tests revealed that the mice in the non-stress group did not differ at all with respect to MHPG concentrations, but the vehicle-treated mice in the stress group demonstrated an increase, and this effect was largely reduced by the CRH antagonist. Levels of DA in the PVN also varied as a function of the stressor, $F(1,22) = 6.73$, $p < .05$, and as a function of the drug treatment, $F(1,22) = 6.57$, $p < .05$, with no Drug x Stressor interaction. Follow-up tests revealed little difference between the vehicle- and drug treated mice in the non-stress group, but the vehicle-treated mice in the stress group demonstrated increased DA levels, and the drug attenuated this effect back to levels comparable to the non-stressed mice, $p < .05$. There was a trend towards a stress-induced increase in DOPAC and 5-HIAA accumulation and a drug-induced reduction, but these effects were shy of statistical significance (see Figure 9 below).

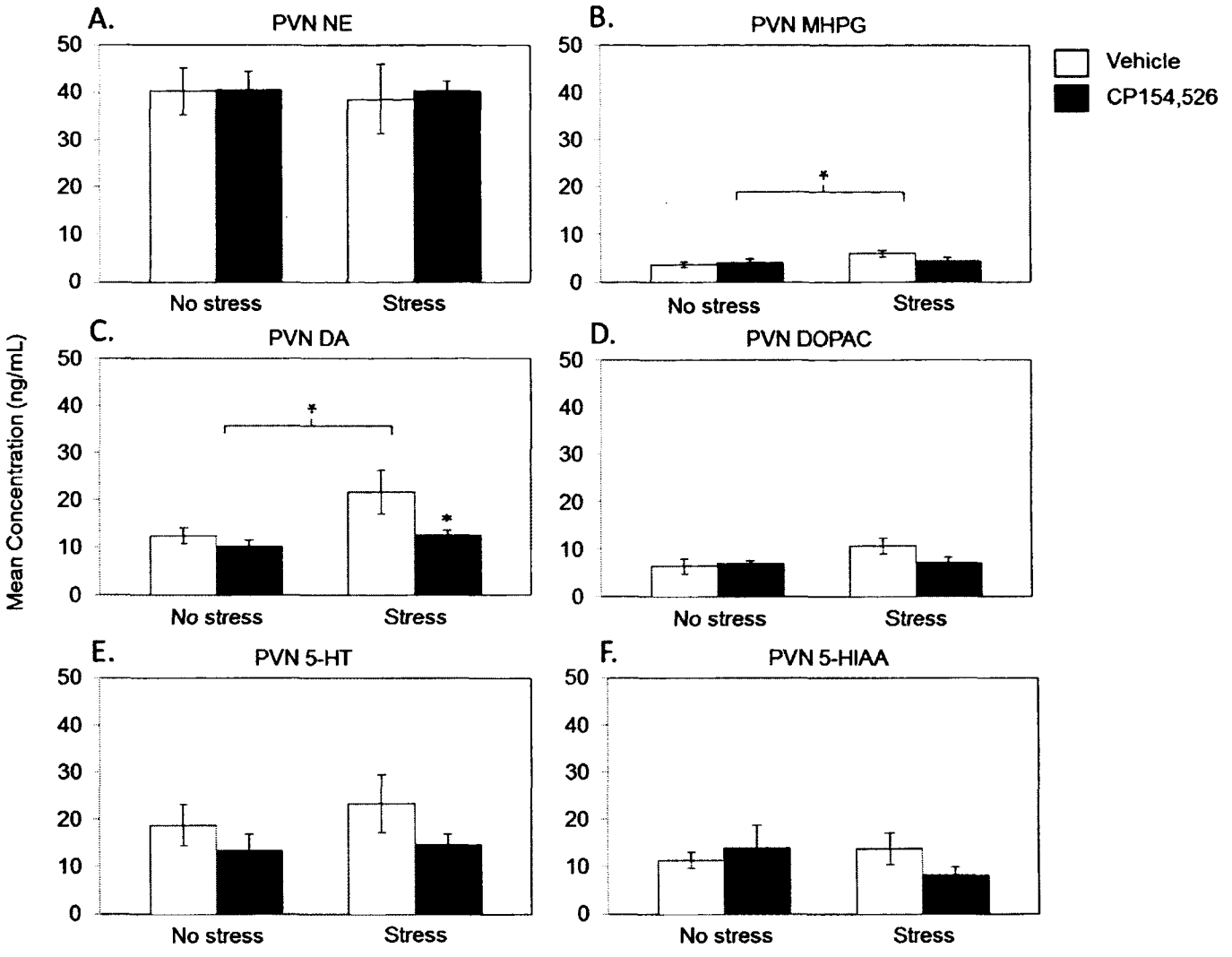

Figure 9. Mean + SEM monoamine variations in the periventricular nucleus of the hypothalamus (PVN) as a function of the CP 154,526 treatment (10 mg/kg, i.p.) that mice received. There was a significant but modest effect of stress on MHPG accumulation within the PVN (see Panel B). Also, both the drug and stressor treatments provoked significant variations of DA within the PVN (see Panel C).

In the CeA, MHPG concentrations varied as a function of the Drug x Stressor interaction, $F(1,26) = 4.62$, $p < .05$, but not by either treatment alone. Follow-up tests revealed no differences between mice in the non-stressed group. However, there was a large increase in MHPG concentrations among the vehicle-treated mice in the stress group, and this effect was attenuated by the CRH antagonist, $p < .05$. Levels of NE in the CeA also varied as a function of the drug treatment, $F(1,26) = 8.44$, $p < .01$, but not by the stressor. With respect to the NE variations in the CeA, follow-up tests revealed the same trend as in the MHPG concentrations, with a stress-induced increase in the vehicle-treated mice and a reduction by the drug, $p < .05$. Although concentrations of DOPAC within the CeA did not vary as a function of the stressor, they did as a function of the drug treatment, $F(1,26) = 7.93$, $p < .01$. Again, there was a trend of a stress-induced increase among the vehicle-treated mice (although non-significant), and a reduction by the drug. In fact, the same trend was also apparent for 5-HIAA concentrations within the CeA, as they modestly varied as a function of the drug treatment, $F(1,26) = 3.25$, $p = .08$, although not significant (see Figure 10 below).

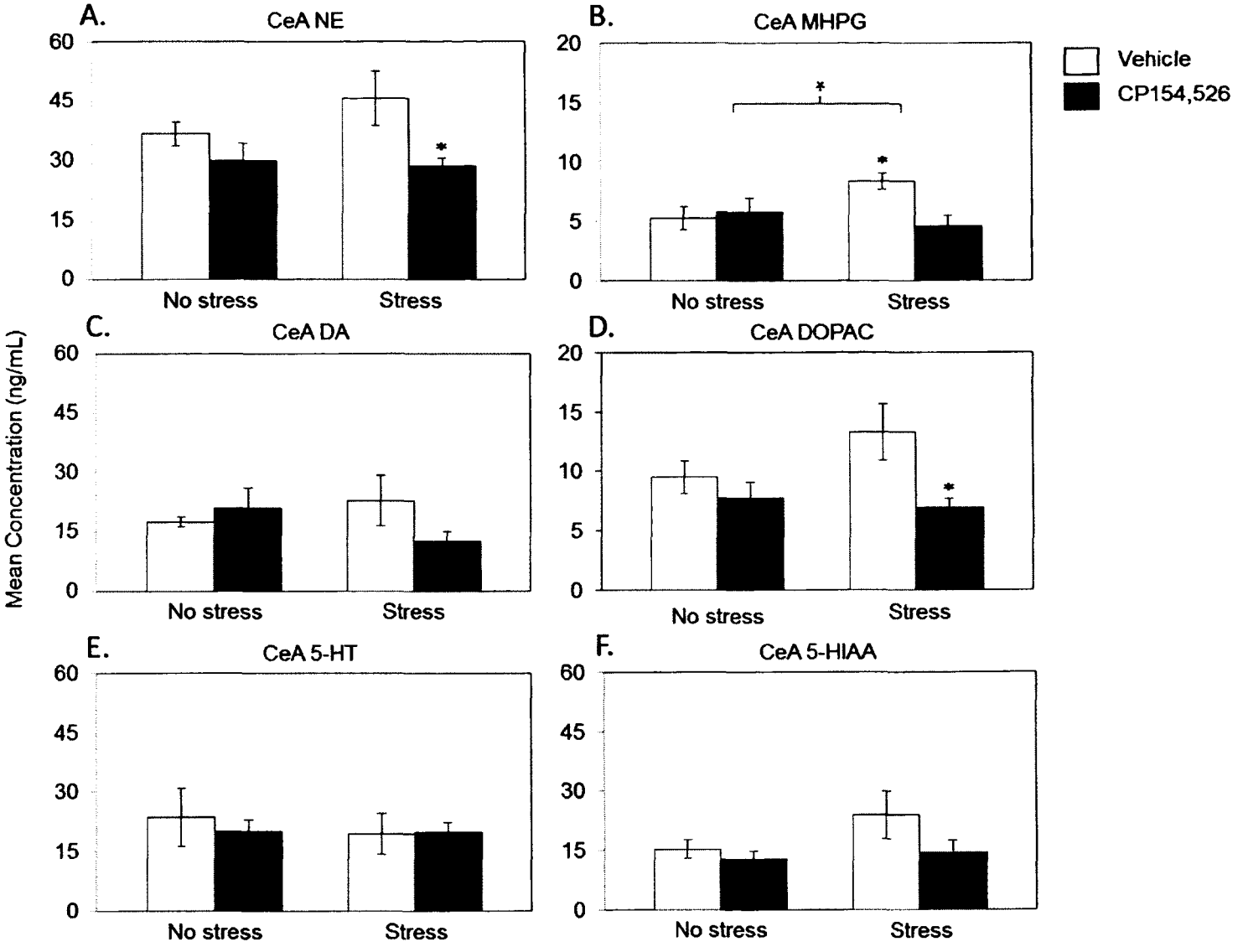

Figure 10. Mean + SEM monoamine variations in the central amygdala (CeA) as a function of the CP 154,526 treatment (10 mg/kg, i.p.) that mice received. NE levels significantly varied as a function of the drug (see Panel A), and there was also a significant Drug x Stressor interaction on MHPG concentrations within the CeA (see Panel B). Further, DOPAC concentrations varied as a function of the drug (see panel D).

4. Discussion

A brief acute psychosocial stressor comprising social defeat induces elevated HPA axis activity and brain monoamine variations, and ought to increase anxiety reflected by diminished entries into the open arms of a plus maze. However, atypical behaviour may be apparent among mice tested in the EPM shortly after a social stressor, possibly owing to increased impulsivity or arousal that might be elicited (Jacobson-Pick et al., 2010, 2011). It was, in fact, observed in the present investigation that mice that had experienced a social defeat stressor exhibited shorter latencies to enter the open arms of the EPM, coupled with signs of hyper-arousal. Although it is uncertain whether the increased responsiveness of the stressed animals reflects impulsivity, Jacobson-Pick et al. (2010, 2011) attributed similar effects to elevated impulsivity that could persist for several weeks before signs of anxiety became apparent. Such an outcome was not restricted to performance in an elevated plus maze, nor was it found only in response to a defeat stressor. In this regard, it has been reported that, following uncontrollable foot shock, a period of hyper-arousal occurs soon afterward so that animals show invigorated responding in a forced swim test. Only after 24 hr is the arousal diminished, giving way to the depressive-like profile in the stressed mice (Prince & Anisman, 1984; Prince et al., 1986).

Both 5-HT and dopamine in the PFC have been shown to differentially modulate impulsive behaviours (Eagle & Baunez, 2010; Gaalen, 2006) and the nucleus accumbens is a DAergic forebrain target of serotonergic neurons arising from the DRN (Abrams et al., 2005). Inasmuch as variations of DOPAC concentrations and in 5-HT levels were observed in the PFC following the CRH treatment, it raises the possibility that the altered behavioural changes elicited by the stressor (experiments 3 and 5) might be related to impulsivity. Unfortunately, it is difficult

to disentangle the effects of stressors on anxiety, arousal and anxiety, although analyses of the temporal changes of these behaviours as well as the neurochemical changes elicited by stressors might be helpful in this regard. Furthermore, as each of these forebrain-related behaviours can be instigated by stressors, it will be necessary to conduct more discrete biochemical analyses involving different aspects of the medial prefrontal cortex (e.g., differentiating the role of the anterior cingulate cortex from other regions, such as the sulcus and later prefrontal cortex).

Since a single social defeat exposure activates 5-HT neurons in the DRN, coupled with the fact that the DRN projects to the forebrain and is targeted by CRH (Thomas et al. 2003), it was believed that CRH receptors located in the DRN might mediate various aspects of anxiety-related behaviour through 5-HT pathways to the PFC (da Veiga et al., 2011). Indeed, the results of the present studies confirmed that CRH infusion into the DRN altered 5-HT functioning in the PFC and affected anxiety-like, as well as, impulsive behaviour. Further, antagonism of CRH_1 receptors, and modestly by CRH_2 receptors, reversed many of the stress-induced monoamine variations and aspects of both anxiety- related- and impulsive behaviours in response to the bullying manipulation, as seen in the EPM.

Generally, acute stressors increased HPA axis activity as well as increased monoamine variations (and their metabolites) in the forebrain. In the present studies, we sought to determine whether CRH receptor activation or blockade of this site of 5-HT cell bodies would influence anxiety-like behaviour and the accompanying PFC monoamine variations. Indeed, many of the central monoamine variations induced by the acute bullying manipulation could be attenuated by pre-administration of CRH receptor antagonists given prior to the stressor, depending on the brain region examined. These variations were apparent in the PFC, PVN, and CeA, but

interestingly, not in the hippocampus. However, considering the short delay between the end of the social defeat stressor and the time of sacrifice, the possibility needs to be considered that the lack of monoamine variations observed in the hippocampus might have been apparent at other times. In contrast to the effects seen in the PFC, the treatment had no effect on the hippocampus, and therefore, data were not shown.

We demonstrated that CRH administration into the DRN, much like the behavioural effects produced by stressors, provoked a dose-dependent effect on anxiety-related behaviour, as seen in the EPM (see Experiment 1). Mice that received the CRH treatment exhibited increased latencies to enter the open arms of the plus maze and spent less time there. In parallel, the middle CRH dose (0.750ug) increased 5-HIAA and DOPAC concentrations in the PFC, and increased 5-HIAA accumulation in the hippocampus. As mentioned previously, dopamine and 5-HT in the PFC have been implicated in impulsive decision making and behaviours (Eagle and Baunez, 2010; Gaalen, 2006), and the present results are in line with those findings. Further, we demonstrated that CRH_2 receptor antagonism within the DRN modestly reduced 5-HIAA accumulation in the PFC and reduced the latency to enter the open arms of the EPM. However, this drug effect was only apparent among the mice in the non-stress group, and in contrast, the opposing behavioural outcome was elicited by mice in the stress group. Although these results were puzzling, it can be appreciated that activation of CRH_1 and CRH_2 receptors have been shown to elicit opposing behavioural responses to stress (Ji & Neugebauer, 2007; Zhao et al., 2007), and the functional role of CRH_2 receptors likely depends on specific stressor characteristics. However, it has been suggested that CRH_2 receptors play an important role in aiding recovery from acute stressor exposure (Neufeld-Cohen et al., 2012). Thus, it must be appreciated that acute antagonism of CRH_2 receptors in the DRN may have facilitated anxiety-

like behaviour produced by the social defeat stressor that was used, and possibly explain the opposing behavioural results seen in the EPM in the present investigation (see Experiment 4). In contrast, systemic injection of CP 154,526 attenuated many of the monoaminergic variations that were provoked by the social defeat stressor, and anxiety-like behaviours were reduced (see Experiments 5 and 6). These results were not surprising and are in line with previous research, reporting that the anxiogenic actions of CRH are mediated by CRH_1 receptors (Muller et al., 2003, Nguyen et al., 2006; Sahuque et al., 2006).

Although we focused primarily on the CRH-mediated variations of 5-HT in the PFC as a modulator of anxiety-related behaviour, the possibility exists that urocortin may also be implicated in this respect. Since the urocortin amino acid peptide family (UCN1, UCN2, UCN3) closely resembles the homology of CRH and can act on CRH receptors, they too may have contributed to the behavioural and monoamine outcomes produced by the social defeat stressor. Urocortin binds with roughly equal affinity to both CRH receptor subtypes, but has a much higher affinity for the CRH_2 receptor than does CRH, and exists in regions of the brain that are distinct from CRH (Ryabinin et al., 2012). Indeed, urocortin mRNA is densely expressed in the dorsal raphe nucleus (DRN), a known mediator of various aspects of the behavioural response to uncontrollable stressors (Hammack et al. 2002), and it has been proposed that the urocortin-CRH_2 system might comprise a separate transmitter system related to CRH (Arborelius et al. 1999; Hammack, 2003). In the main, the urocortin-CRH_2 mechanisms have been linked to the pathophysiology of various psychiatric disorders, but their interaction in mediating stress-related behaviours remains unclear. However, considering the intricate relationship between CRH and urocortin, it remains a possibility that the results of the present investigation may, in fact, have been influenced by urocortin.

Other limitations of the present investigation pertain to the use of isoflurane during drug infusion. Although mice were given time to recover, this can obviously affect behaviour and potentially influence the behavioural results. Further, the behavioural testing was a brief and acute test in the elevated plus maze, and it would have perhaps been beneficial to have re-tests in order to tease out what behaviours were due to hyper-arousal/impulsivity, and which may have been due simply to anxiety-related behaviours. Lastly, owing to the stressor, the open field data were highly variable and were deemed not reliable (this is depicted in Figure 2).

It seems that CRH_1 and CRH_2 receptors might both contribute to mood states and behaviour, but they likely do so through different processes. As previously mentioned, the phenotypes of mice deficient in CRH_1 receptors is characterized by an impaired stress response and decreased anxiety-like behaviour. In contrast, mice deficient in CRH_2 receptors display hypersensitivity to stressors and increased anxiety-like behaviour (Bale, 2002). According to Kishimoto et al. (2000), this is not due the HPA axis dysregulation, but to impaired responses in specific brain regions that are involved in autonomic and emotional function. It is well established that CRH_1 receptors play a critical role in coordinating the endocrine response to stress through HPA axis activation, and that CRH_2 receptors mediate various aspects of behavioural adaptation to stressors, possibly through interactions with other neurotransmitters. However, the functional role of CRH receptors in modulating anxiety-related behaviours through forebrain monoamine variations remains somewhat unclear. Although CRH receptors have been implicated as therapeutic targets for anxiety and depression, before this can be accomplished, it will be necessary to determine the respective contributions of the receptor subtypes to these behaviours.

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