Interactions of TCAP-1 and Endocannabinoids with Corticotropin-Releasing Factor in Mediating Cocaine- and Anxiety-Related Behaviour

by

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Abstract

The neuropeptide, corticotropin-releasing factor (CRF), plays a critical role in the central regulation of various stress-related behaviours, including those unique to subjects with prior cocaine experience. The three series of experiments presented in this dissertation explored the role of two neurochemical systems, the teneurin C-terminal associated peptides (TCAP) and the endocannabinoids (eCBs), in several cocaine- and anxiety-related behaviours induced or mediated by CRF.

The first series of experiments examined the effects of TCAP-1 on the reinstatement of cocaine seeking and expression of cocaine-induced behavioural sensitization. Repeated (5-day), but not acute, TCAP-1 treatment blocked the reinstatement of cocaine seeking induced by central injections of CRF. TCAP-1 was, however, without effect on footshock- or cocaine-induced reinstatement. Repeated TCAP-1 further interfered with the expression of behavioural sensitization to a CRF, but not a cocaine, challenge. These findings suggest that TCAP-1 normalizes CRF signaling dysregulated by cocaine exposure to interfere in the subsequent effects of CRF on cocaine-related behaviours.

A parallel series of experiments investigated the role of eCB signaling at CB_1 receptors in the reinstatement of cocaine seeking and cocaine-sensitized locomotion. Pretreatment with

the CB_1 receptor antagonist, AM251, selectively interfered with CRF-, but not footshock- or cocaine-induced reinstatement. AM251 further blocked the expression of behavioural sensitization induced by challenge injections of both CRF and cocaine. These findings reveal a mediating role for CB_1 receptor transmission in the effects of CRF on cocaine-related behaviours.

A final series of experiments examined the role of CB_1 receptor transmission in the behavioural anxiety induced by central injections of CRF, and by withdrawal from chronic cocaine exposure. AM251, although itself anxiogenic, reversed anxiety induced by CRF and cocaine withdrawal. Furthermore, AM251 elevated plasma corticosterone levels, indicative of increased HPA axis activity, irrespective of CRF treatment or cocaine withdrawal. These findings suggest that CRF- and cocaine withdrawal-induced anxiety are mediated, at least in part, by CB_1 receptor transmission, independent of HPA axis regulation.

The collective findings are discussed within a framework of CRF-TCAP-eCB interactions, wherein TCAP-1 and AM251 are proposed to act in parallel to modulate amygdalar CRF transmission, and thus regulate the expression of cocaine- and anxiety-related behaviours.

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Kupferschmidt DA, Lovejoy DA, Rotzinger S, Erb S (2011) Teneurin C-terminal associated peptide-1 blocks the effects of corticotropin-releasing factor on reinstatement of cocaine seeking and on cocaine-induced behavioural sensitization. British Journal of Pharmacology 162: 574-583.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2-arachidonoylglycerol. 2-AG

Activator protein-1. . . . . .AP-1

Activating transcription factor-1. . . . .ATF1

Adenosine triphosphate. . . . . .ATP

Adrenocorticotropic hormone. . . . . .ACTH

α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid. . . . . AMPA

Analysis of variance. . . . . .ANOVA

Anandamide. . . . . .AEA

Basolateral amygdala. . . . . BLA

Bed nucleus of the stria terminalis. . .BNST

β-dystroglycan. . . . . .β-DG

Brain-derived neurotrophic factor. . .BDNF

Cannabinoid. . . . . CB

Central nucleus of the amygdala. . . . . .CeA

Complementary deoxyribonucleic

acid. . . . . .cDNA

Conditioned place preference. . . . . CPP

Corticotropin releasing factor. . . . . .CRF

Cyclic adenosine monophosphate. . . cAMP

cAMP response element-binding. . . .CREB

Dimethyl-sulfoxide. . . . . .DMSO

Dopamine. . . . . DA

Elevated plus maze. . . . . .EPM

Endocannabinoid. . . . . .eCB

Extinction. . . . . EXT

Extracellular signal-regulated kinase. .ERK

Fixed-ratio. . . . . FR

γ-aminobutyric acid. . . . . GABA

Intracerebroventricular. . . . . .i.c.v.

Intraperitoneal. . . . . i.p.

Intravenous. . . . . i.v.

N-methyl-D-aspartic acid. . . . . .NMDA

Hypothalamic-pituitary-adrenal. . . . . .HPA

Lateral tegmental nuclei. . . . . .LTN

Medial prefrontal cortex. . . . . .mPFC

Messenger ribonucleic acid. . . . . .mRNA

Mitogen-activated protein. . . . . MAP

Mitogen-activated protein kinase

kinase. . . . . .MEK

Nucleus accumbens. . . . . .NAc

Paraventricular nucleus. . . . . PVN

Periaqueductal gray. . . . . PAG

Prefrontal cortex. . . . . PFC

Ribosomal S6 kinase. . . . . .RSK

Saline. . . . . .SAL

Standard error of the mean. . . . . .SEM

Teneurin c-terminal associated peptide. . . . . TCAP

Transient receptor potential vanilloid type 1. . . . . TRPV1

Tropomyosin receptor kinase B. . . . . .TrkB

Vehicle. . . . . .VEH

Ventral tegmental area. . . . . .VTA

CHAPTER 1

General Introduction

CHAPTER 1

General Introduction

Stress has long been considered an important factor contributing to an increased risk of cocaine abuse and relapse in humans (Brownell et al., 1986; Sinha, 2001). Retrospective reports indicate that the negative mood and heightened anxiety states that characterize the initial phase of cocaine withdrawal (Gawin and Kleber, 1986) often impede abstinence in regular drug users (Bradley et al., 1989; Gawin and Ellinwood, 1989). Following protracted drug-free periods, cocaine abusers continue to attribute their pronounced drug craving and relapse to stressful situations and negative moods (McKay et al. 1995).

Evidence from controlled clinical laboratory experiments further supports a role for stress in cocaine craving and relapse. For example, exposure to stressful stimuli, such as guided stressful imagery (Sinha et al., 1999; Fox et al., 2005), social stress tasks (Moran-Santa Maria et al., 2010), and intravenous (i.v.) treatment with the stress-related neuropeptide, corticotropin-releasing factor (CRF; Back et al., 2010; Moran-Santa Maria et al., 2010), have been shown to induce drug craving in cocaine-dependent individuals. Moreover, subjective ratings of stress induced by such stimuli positively correlate with measures of cocaine craving (Moran-Santa Maria et al., 2010; Brady et al., 2009). Cocaine abusers also exhibit heightened responsivity to stressful stimuli. Indeed, individuals with a history of cocaine use report more pronounced emotional arousal, anxiety, and drug craving following exposure to various laboratory stressors (e.g. guided imagery, i.v. CRF), relative to drug-naïve subjects (Brady et al., 2009; Chaplin et al., 2010; Moran-Santa Maria et al., 2010). This heightened stress responsivity is thought to contribute to the persistence of cocaine use in dependent individuals (Chaplin et al., 2010). In fact, the degree of anxiety and cocaine craving seen in response to stressful imagery or CRF treatment associate positively with the risk of subsequent cocaine relapse (Sinha et al., 2006; Back et al., 2010).

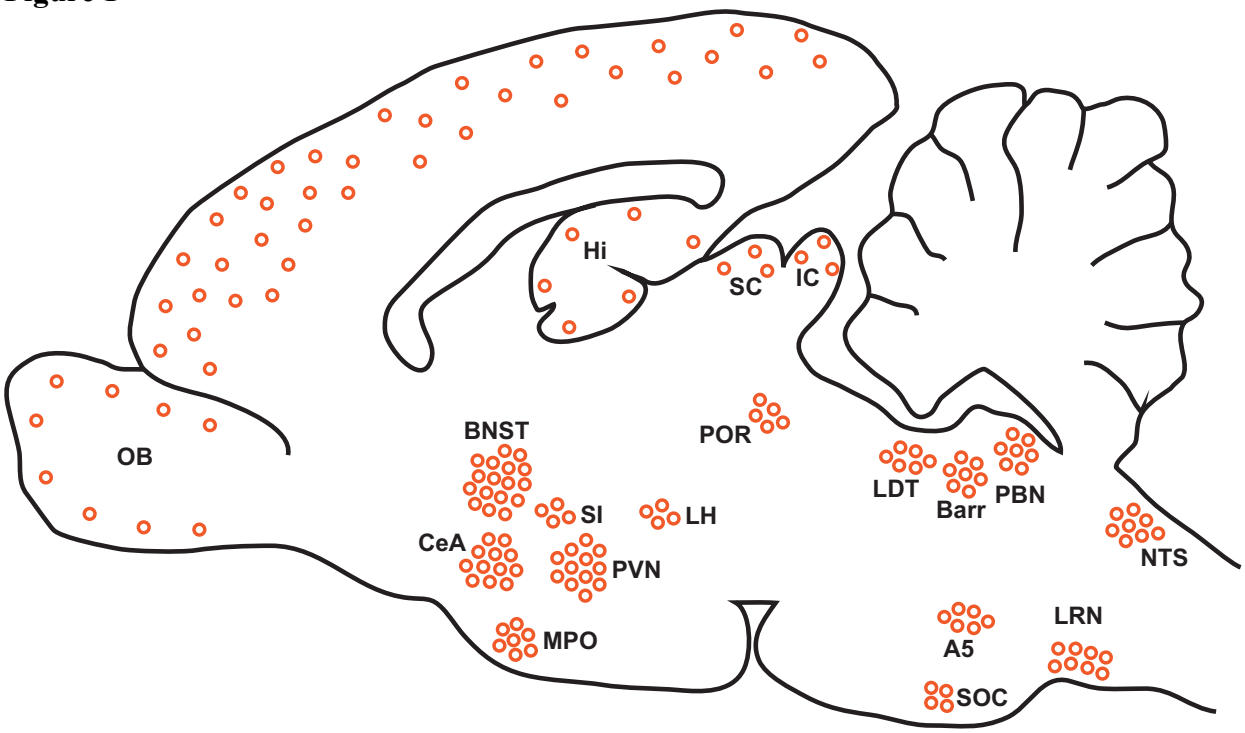
Over the past two decades, considerable preclinical research has been carried out to determine the neurobiological mechanisms underlying the relationship between stress and cocaine dependence. A major emphasis of this work has been on the role of the principle stress-related neurochemical system, CRF, and systems with which CRF interacts, in mediating stress-related behaviours in subjects with prior cocaine experience (see below).

The present dissertation is comprised of experiments carried out to explore the role of two neurochemical systems, the teneurin C-terminal associated peptides (TCAP) and the endocannabinoids (eCBs), in several cocaine- and anxiety-related behaviours induced, or known to be mediated, by CRF. These behaviours, which include long-term reinstatement of cocaine seeking, cocaine-induced behavioural sensitization, and cocaine withdrawal-induced anxiety, will be described in Sections 1.3. The TCAP and eCB systems, and what is known about their interactions with the CRF system, will be described in Sections 1.4 and 1.5. First, however, a general characterization of the central CRF systems will be given.

1.1. Corticotropin-releasing Factor

CRF, a 41-amino acid peptide, plays a critical role in coordinating the overall stress response by mediating many endocrine, autonomic, neurochemical, and behavioural responses to stressors (Dunn and Berridge, 1990). First characterized by Vale and colleagues (1981), CRF is part of a larger family of related peptides, including urocortin I, II, and III, and CRF binding protein (Bale and Vale, 2004). CRF is distributed widely throughout the brain, with notable immunoreactivity in the hypothalamus, extended amygdala (e.g. central nucleus of the amygdala [CeA], bed nucleus of the stria terminalis [BNST]), neocortex, olfactory bulb, midbrain (e.g. ventral tegmental area [VTA]), cerebellum, and brainstem (e.g. locus coeruleus, nucleus of the solitary tract) (See Figure 1; Swanson et al., 1983; 1993; Rodaros et al., 2007).

Figure 1



Schematic of the distribution of CRF cell bodies in the rat brain.

Abbreviations: Barrington's nucleus (Barr), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), hippocampus (Hi), inferior colliculus (IC), lateral hypothalamus (LH), lateral reticular nucleus (LRN), lateral tegmental nucleus (LTG), medial preoptic area (MPO), nucleus of the solitary tract (NTS), olfactory bulb (OB), parabrachial nucleus (PBN), paraventricular nucleus of the hypothalamus (PVN), perioculomotor raphe (POR), substantia innominate (SI), superior colliculus (SC), superior olivary nucleus (SON). Adapted from Sawchenko et al. (2010).

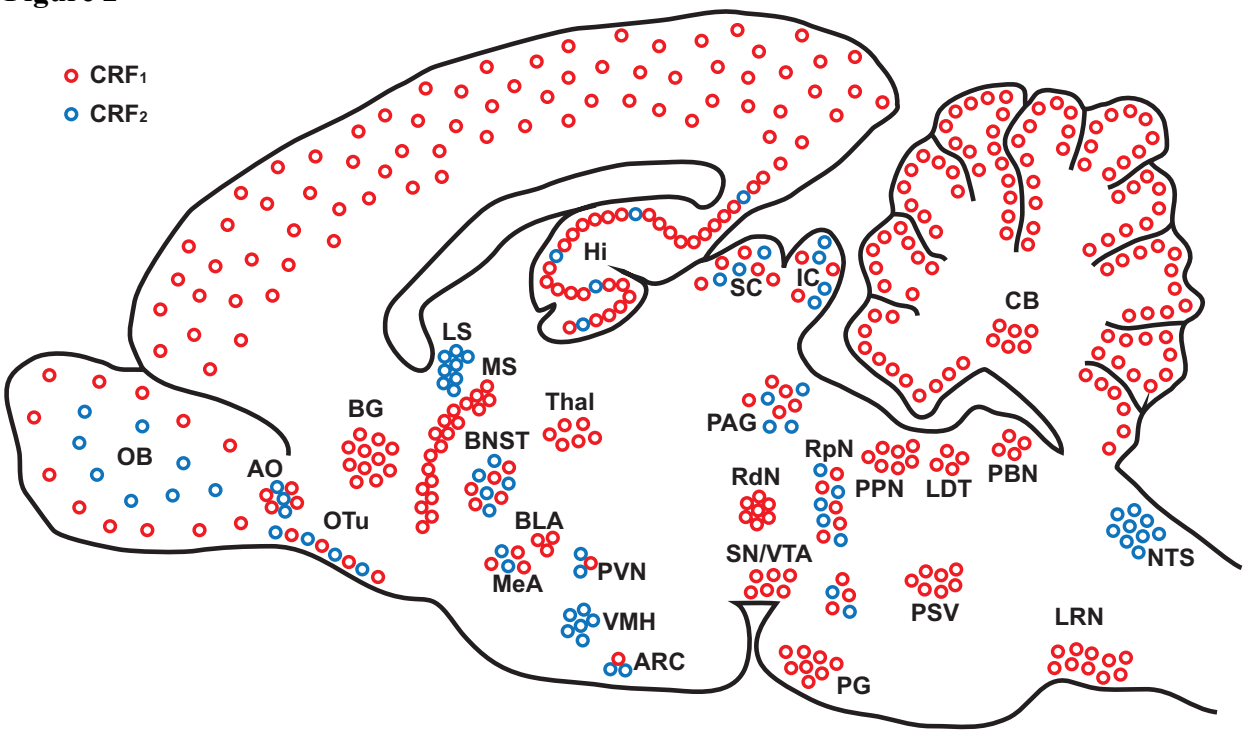
CRF binds and activates two G protein-coupled receptors, CRF_1 and CRF_2 (Chen et al., 2000; Dieterich et al., 1997). Although both receptors primarily act through G_s-proteins and adenylate cyclases to increase cyclic adenosine monophosphate (cAMP) production, CRF receptors can interact with other G protein systems, including G_q, G_i, G_o, $G_{il/2}$, and G_z (Grammatopoulos et al., 2001). As such, CRF receptors modulate in a tissue- and dose-dependent manner various signaling pathways and kinases, including phosphokinases A, B and C, mitogen-activated protein (MAP) kinases, and intracellular calcium concentrations. CRF_1 receptors, the most abundant and highest affinity receptors for CRF in the brain (Perrin et al., 1995), are densely expressed in the neocortex, cerebellum, olfactory bulb, amygdala, medial septum, hippocampus, and pituitary, whereas CRF_2 receptors display more limited central expression that is most pronounced in the lateral septum and ventromedial nucleus of the hypothalamus (See Figure 2; Potter et al., 1994; Chalmers et al., 1995; Primus et al., 1997).

In its endocrine role, CRF initiates a well-characterized cascade of events involving activation of the hypothalamic-pituitary-adrenocortical (HPA) axis. Specifically, CRF is released from neurosecretory cells of the paraventricular nucleus (PVN) of the hypothalamus into the median eminence in response to stress. Upon binding to CRF_1 receptors in the anterior pituitary, CRF induces the release of adrenocorticotropic hormone (ACTH) into general circulation, which in turn stimulates the release of corticosterone in rodents (cortisol in humans) from the adrenal cortices (Dunn and Berridge, 1990; Sarnyai et al., 2001). Collectively, these processes play a critical role in mobilizing energy stores and modulating blood pressure in response to stress (Herman et al., 2003).

1.2. CRF and Stress-related Behaviour

Independent of its endocrine role, CRF acts as a neurotransmitter on CRF_1 and CRF_2 receptors in extrahypothalamic brain regions to mediate various physiological and behavioural

Figure 2



Schematic of the distribution of CRF_1 and CRF_2 receptors in the rat brain.

Abbreviations: anterior olfactory nucleus (AO), arcuate nucleus of the hypothalamus (ARC), basal ganglia (BG), basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST), cerebellum (CB), hippocampus (Hi), inferior colliculus (IC), lateral reticular nucleus (LRN), lateral septum (LS), laterodorsal tegmental nucleus (LDT), medial septum (MS), medial nucleus of the amygdala (MeA), nucleus of the solitary tract (NTS) olfactory bulb (OB), olfactory tubercle (OTu), parabrachial nucleus (PBN), paraventricular nucleus of the hypothalamus (PVN), pedunculopontine nucleus (PPN), periaqueductal gray (PAG), pontine gray (PG), primary sensory nucleus of the trigeminal nerve (PSV), raphe nucleus (RpN), red nucleus (RdN), substantia nigra (SN), superior colliculus (SC), thalamus (Thal), ventral tegmental area (VTA), ventromedial nucleus of the thalamus (VMH). Adapted from Sawchenko et al. (2010).

responses to stress (Sarnyai et al., 2001). For example, intracerebroventricular (i.c.v.) injections of CRF induce anxiety-like behaviours in rodents (e.g. Baldwin et al., 1991; Spina et al., 2002) that are unaltered by manipulations of HPA activity, such as hypophysectomy, pretreatment with the glucocorticoid receptor agonist, dexamethasone, or PVN lesions (Eaves et al., 1985; Britton et al., 1986; Berridge and Dunn, 1989; Liang et al., 1992). Furthermore, i.c.v. administration of CRF receptor antagonists counters various behavioural responses to stressors (e.g. Menzaghi et al., 1994; Spina et al., 2000).

The extended amygdala circuitry comprises a major component of the extrahypothalamic CRF system. First characterized by de Olmos and colleagues (1985), the extended amygdala is composed of a continuum of neurons in the basal forebrain connecting the CeA, BNST, and nucleus accumbens (NAc) shell. These structures are associated on the basis of their shared neurochemical composition, morphology, and connectivity (Alheid and Heimer, 1988). As mentioned, the CeA and BNST are dense with neurons immunoreactive for CRF and CRF mRNA, particularly in their lateral subregions (Potter et al., 1994; Swanson et al., 1983; Sakanaka et al., 1986). These neurons target various hypothalamic, brainstem and midbrain areas, including the VTA (Rodaros et al., 2007; Sakanaka et al., 1986). Both the CeA and BNST also contain intrinsic networks of CRF neurons (Uryu et al., 1992; Phelix and Paull, 1990; Veinante et al., 1997). Moreover, the CeA provides an important source of CRF to the lateral BNST (Sakanaka et al., 1986). The BNST expresses both CRF_1 and CRF_2 receptors, whereas the CeA expresses only CRF_1 receptors (Bittencourt and Sawchenko, 2000; Chalmers et al., 1995; Van Pett et al., 2000; Chen et al., 2000). Principal inputs to the CeA and BNST include the basolateral amygdala (BLA), hypothalamus, insular and medial frontal cortices, and VTA (Heimer and Alheid, 1991; Alheid et al., 1995; Savander et al., 1995; Pitkänen, 2000).

Considerable evidence implicates CRF signaling within the extended amygdala in the expression of anxiety and other stress-related responses. Indeed, local infusion of CRF and CRF

receptor agonists into the rat BNST inhibits social interaction (Lee et al., 2008), reduces open arm time and entries in the elevated plus maze (EPM; Sahuque et al., 2006), enhances acoustic startle amplitude (Lee and Davis, 1997), increases retention of an inhibitory avoidance task (Liang et al., 2001), and produces conditioned aversion to environments previously associated with the CRF infusion (Sahuque et al., 2006). Furthermore, excitotoxic lesions of the BNST, and intra-BNST infusion of the non-selective CRF receptor antagonist, α-helical CRF_{9-41}, block the enhanced acoustic startle response seen in rats following i.c.v. CRF administration (Lee and Davis, 1997).

Although direct manipulation of CRF transmission within the CeA is largely without effect on anxiety-like behavior (for review, see Davis et al., 2010), the CeA provides an important source of CRF to the BNST to mediate such behaviour. For example, Jasnow and colleagues (2004) found that socially defeated hamsters given unilateral lesions of the CeA and infusions of the non-selective CRF receptor antagonist, D-Phe CRF_{12-41}, into the contralateral BNST displayed significantly less submissive behaviour during a subsequent social interaction than those given only one such manipulation. These results suggest that stress (i.e. conditioned defeat stress) can activate CRF neurons projecting from the CeA to the BNST to mediate stress-related behaviour. Consistent with these findings, conditional knockdown and overexpression of CRF mRNA in the CeA, using local lentiviral vector administration, attenuated and exacerbated, respectively, the anxiety-like behaviour seen in mice in response to restraint stress (Regev et al., 2011).

Another brain region intimately associated with structures of the extended amygdala, and implicated in the regulation of behavioural anxiety, is the BLA. Anatomically, the BLA is positioned to receive information regarding salient stimuli from its substantial cortical and thalamic inputs, and orchestrate behavioural responses to these stimuli via projections to the CeA, BNST, NAc, hippocampus, and prefrontal cortex (PFC) (Pitkänen, 2000; Dong et al.,

2001; Sah et al., 2003; Knapska et al., 2007). In particular, neurons projecting from the BLA to the CeA have been implicated in the regulation of many endocrine, autonomic, and behavioural responses to stress (Davis, 1997; Millan, 2003; Walker al., 2009). The balance of excitatory and inhibitory (from local γ-aminobutyric acid [GABA] interneurons) input to these BLA projection neurons plays a critical role in the regulation of behavioural responses to stress, including anxiety. For example, local infusions of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and $GABA_A$ receptor antagonists into the BLA reduce (Sajdyk and Shekhar, 1997; Walker and Davis, 1997) and induce (Sanders and Shekhar, 1995), respectively, anxiety-like behaviour in the social interaction and acoustic startle paradigms.

Consistent with the dense CRF_1 receptor expression in the BLA (Bittencourt and Sawchenko, 2000; Chen et al., 2000), and the excitatory effects of CRF_1 receptor transmission on BLA projection neurons (Rainnie et al., 2004; Ugolini et al., 2008; Giesbrecht et al., 2010), the BLA has been implicated in the effects of CRF signaling on anxiety-like behaviour. For example, intra-BLA infusions of CRF and CRF receptor agonists induce anxiety and aversion in the social interaction and conditioned place avoidance paradigms (Sajdyk et al., 1999; Gehlert et al., 2005; Sajdyk et al., 2006; Spiga et al., 2006; Lee et al., 2008). Moreover, pharmacological inactivation of the BLA has been shown to block the potentiated startle response to i.c.v. CRF administration (Walker et al., 2009).

Taken together, the results from preclinical studies provide substantial support for the notion that CRF signaling within extrahypothalamic brain regions mediates numerous behavioural responses to stress. Considerable evidence also indicates that central CRF signaling plays an important role in many behavioural effects of stress that are unique to subjects with prior exposure to drugs of abuse like cocaine. Indeed, as will be discussed, exposure to cocaine dysregulates CRF systems such that many subsequent cocaine-related behaviours come to be induced, or mediated, by CRF. The following sections will outline evidence for a role of CRF

in the effects of stress on three cocaine-related behaviours that form the basis of experiments described in this dissertation: the reinstatement of cocaine seeking, cocaine-induced behavioural sensitization, and cocaine withdrawal-induced anxiety.

1.3. CRF and Cocaine-related Behaviour

1.3.1. CRF and Reinstatement of Cocaine Seeking

Since their original characterization by Stretch and colleagues (1971), animal models of relapse known as reinstatement procedures have been used extensively to investigate the role of stress in cocaine relapse. In a typical reinstatement procedure, animals are trained to perform an operant behaviour (i.e. lever press) for an i.v. infusion of a reinforcing drug like cocaine. The drug-reinforced behaviour is subsequently extinguished by discontinuing the response-contingent drug infusion. Reinstatement of the extinguished behaviour is then induced by exposure of the animal to: (1) non-contingent administration of the previously self-administered drug (or one like it); (2) cues previously association with drug taking; or (3) acute physical, environmental, or pharmacological stress. The number of non-reinforced operant responses made in response to, or 'reinstated' by, such stimuli is used as a measure of drug seeking (Shaham et al., 2003).

The first stressor shown to induce the reinstatement of drug seeking in rats with a history of cocaine self-administration was exposure to mild, intermittent footshocks (Erb et al., 1996). This effect has been observed using modified schedules of reinforcement, drug dosages, footshock parameters, and rat strains (Ahmed and Koob, 1997; Ahmed et al., 2000; Mantsch and Goeders, 1999), and in rats with self-administration histories other than cocaine, including heroin, alcohol, and nicotine (Shaham and Stewart, 1995; Lê et al., 1998; Buczek et al., 1999). In addition to footshock, exposure to acute, 1-day food deprivation stress reliably reinstates drug-seeking behavior (Shalev et al., 2000; 2003; Funk et al., 2005). Administration of various

pharmacological stressors, including the corticosterone synthesis inhibitor, metyrapone (Shaham et al., 1997), and the anxiogenic, α2-adrenoreceptor antagonist, yohimbine (Shepard et al., 2004; Lee et al., 2004; Lê et al., 2005; Kupferschmidt et al., 2009), also induces robust reinstatement of drug-seeking behaviour.

Given the critical role that CRF plays in various behavioural responses to stress, it is not surprising that it has been a primary focus of studies investigating the neurobiology of footshock-induced reinstatement of cocaine seeking. Indeed, initial studies revealed that i.c.v. pretreatment with the CRF receptor antagonist, D-Phe CRF_{12-41}, or systemic pretreatment with the CRF_1 receptor antagonist, CP-154,526, blocked footshock-induced reinstatement of cocaine seeking (Erb et al., 1998; Shaham et al., 1998). In addition, i.c.v. injections of CRF itself reinstated cocaine-seeking behaviour (Erb et al., 2006a; Mantsch et al., 2008).

CRF signaling may also be involved in the reinstatement of cocaine seeking induced by non-contingent priming injections of cocaine, although the findings to date are equivocal. In the original study demonstrating a definitive role for CRF in footshock-induced reinstatement of cocaine seeking, Erb and colleagues (1998) reported that cocaine prime-induced reinstatement was minimally attenuated by i.c.v. pretreatment with the CRF receptor antagonist, D-Phe CRF_{12-41}. In another study, however, systemic pretreatment with the CRF_1 receptor antagonist, CP-154,526, dose-dependently attenuated reinstatement induced by a priming injection of cocaine (Przegaliński et al., 2005). The reasons for these discrepant findings are unclear, but may include differences in the schedules of reinforcement employed during self-administration (fixed ratio [FR]-1 vs. 5), the durations of self-administration (10-14 vs. 18 days) and extinction training (5-14 vs. 10 days), the pharmacological properties of the two CRF receptor antagonists (non-selective vs. CRF_1 selective), or their routes of administration (i.c.v. vs. i.p.).

The CRF signaling that mediates footshock-induced reinstatement is thought to be extrahypothalamic and HPA-independent given that footshock, despite its robust activation of

the HPA axis (Gispen et al., 1973; Swenson and Vogel, 1983), induces reinstatement of drug seeking even in animals with compromised adrenal function (Shaham et al., 1997; Erb et al., 1998; Lê et al., 2000; but see Mantsch and Goeders, 1999). For example, Erb and colleagues (1998) demonstrated that although footshock-induced reinstatement is impaired in adrenalectomized rats, it is restored in those in which basal levels of corticosterone are replaced. These results suggest that although basal levels of corticosterone play a permissive role in footshock-induced reinstatement of cocaine seeking, a stress-induced rise in corticosterone is not necessary to achieve the effect.

In a series of experiments using local pharmacological manipulations, Erb and colleagues localized the effects of CRF on footshock-induced reinstatement to the BNST and CeA. In one study, Erb and Stewart (1999) demonstrated that bilateral injections of the CRF receptor antagonist, D-Phe CRF_{12-41}, into the BNST, but not the CeA, blocked footshock-induced reinstatement of cocaine seeking. Likewise, local injections of CRF itself into the BNST, but not the CeA, induced the reinstatement of cocaine seeking. Next, using an asymmetric lesion procedure, Erb and colleagues (2001) further demonstrated that functional disconnection of the CRF projection from the CeA to the BNST (Sakanaka et al., 1986) attenuated the effects of footshock stress on the reinstatement of cocaine seeking. Taken together, these results suggest that although activation of CRF receptors in the CeA is not necessary for footshock-induced reinstatement of cocaine seeking, activation of CRF neurons originating in the CeA and projecting to the BNST are importantly involved in the effect.

Recently, CRF transmission in the VTA has been found to mediate the effects of footshock on the reinstatement of cocaine seeking. More specifically, Wang and colleagues (2005a) showed that in rats with a history of cocaine self-administration, CRF in the VTA drives local glutamate and dopamine (DA) release that, in turn, mediates footshock-induced reinstatement of cocaine seeking. In subsequent experiments, selective blockade of CRF_2, but

not CRF_1, receptors in the VTA blocked the action of CRF on footshock-induced reinstatement (Wang et al., 2007). Moreover, VTA perfusion of CRF itself, or CRF_2 receptor agonists with a strong affinity for CRF binding protein, induced reinstatement of cocaine seeking (Wang et al., 2007).

The circuitry mediating footshock-induced reinstatement of cocaine seeking has been extended to include DA transmission in the PFC, presumably downstream of CRF transmission in the VTA. For example, Capriles and colleagues (2003) demonstrated that local administration of D_1-like, but not D_2-like, receptor antagonists into the prelimbic and orbitofrontal cortex blocked footshock-induced reinstatement of cocaine seeking. Consistent with these findings, subsequent experiments showed that administration of the mixed D_1/D_2-like receptor antagonist, fluphenazine, into the dorsal PFC blocked footshock-induced reinstatement (McFarland et al., 2004), as did temporary inactivation of the VTA (McFarland et al., 2004; Wang et al., 2005a).

Corroborating neuroanatomical and functional evidence supports that notion that CRF signaling in the VTA mediates footshock-induced reinstatement via its effects on mesocortical DA transmission. First, *in situ* hybridization and polymerase chain reaction studies indicate the presence of CRF_1 and CRF_2 receptors, respectively, on VTA DA neurons (Bittencourt and Sawchenko, 2000; Chalmers et al., 1995; Van Pett et al., 2000; Ungless et al., 2003). A major source of CRF input to these VTA DA neurons originates from the CeA and BNST, two structures implicated in footshock-induced reinstatement (Rodaros et al., 2007; Swanson et al., 1983). Presumably by activating these structures, footshock stress induces the release of CRF into the VTA (Wang et al., 2005a), where it has been shown, *in vitro*, to potentiate *N*-methyl-D-aspartic acid (NMDA) receptor-mediated excitatory transmission in DA neurons (Ungless et al., 2003), and increase their firing rate (Wanat et al., 2008). Interestingly, the ability of CRF to potentiate excitatory transmission in VTA DA neurons is enhanced in animals with a history of

cocaine exposure (Hahn et al., 2009). In a similar manner, footshock-induced release of CRF into the VTA, an effect that is both necessary and sufficient for the reinstatement of cocaine seeking, also triggers local DA release, but only in rats with a history of cocaine self-administration (Wang et al., 2005a). The CRF-mediated release of local DA, indicative of increased DA neuron activity (Ford et al., 2010), is presumably accompanied by DA release in terminal sites of the mesocortical system, given that both footshock (Sorg and Kalivas, 1993; McFarland et al., 2004) and i.c.v. CRF (Lavicky and Dunn, 1993) have been shown to increase DA release in the PFC. Moreover, recent work has shown that selective genetic deletion of CRF_1 receptors in the VTA attenuates the prefrontal DA release induced by footshock stress (Refojo et al., 2011).

Thus, taken together, the findings to date suggest that CRF transmission in the VTA, presumably resulting from activation of CRF projections originating in CeA and BNST, leads to the downstream release of DA in the PFC. D_1-like receptor transmission in the PFC then activates glutamatergic efferents to the NAc core that mediate the reinstatement of cocaine seeking (Cornish and Kalivas, 2000; Di Ciano and Everitt, 2001; McFarland et al., 2004; Bäckström and Hyytiä, 2007).

1.3.2. CRF and Cocaine-induced Behavioural Sensitization

Behavioural sensitization refers to the enhanced behavioural response to a stimulus following repeated exposure to that stimulus (Robinson and Becker, 1986; Kalivas and Stewart, 1991). This phenomenon has been well characterized with respect to the effects of repeated and intermittent exposure to drugs of abuse, such as cocaine, on locomotor activity (Robinson and Becker, 1986; Kalivas and Stewart, 1991). Indeed, sensitization to the locomotor-activating effects of psychostimulant drugs such as cocaine and amphetamine, as well as morphine, ethanol, nicotine, and delta-9-tetrahydrocannabinol (Joyce and Iversen, 1979; Robinson and

Becker, 1986; Benwell and Balfour, 1992; Cunningham and Noble, 1992; Post et al., 1992; Cadoni et al., 2001), can persist for periods of months after the termination of drug administration (Paulson and Robinson, 1991). Moreover, these sensitized effects of repeated drug exposure are importantly influenced by the contextual cues present during drug exposure and testing phases. In particular, the expression of sensitization is generally enhanced in the presence of stimuli previously associated with drug availability (e.g. Mattson et al., 2008; for review, see Vezina and Leyton, 2009).

It has been argued that drug-induced sensitization of locomotor activity reflects an enhancement of the rewarding and incentive-motivational properties of the drugs (Robinson and Becker, 1986; Robinson and Berridge, 1993). Consistent with this hypothesis, early work by the Simon and Vezina groups demonstrated that rats repeatedly exposed to non-contingent amphetamine injections acquire and maintain subsequent amphetamine self-administration behaviour more readily than drug-naïve rats (Piazza et al., 1989; Pierre and Vezina, 1997). Similar findings have since been reported with cocaine exposure and self-administration (Childs et al., 2006). On the other hand, prior cocaine self-administration potentiates the locomotor response to a subsequent cocaine challenge (Hooks et al., 1994; Phillips and Di Ciano, 1996; Morgan and Roberts, 2004). Studies using the conditioned place preference (CPP) paradigm have provided further evidence that the reinforcing properties of drugs are sensitized following repeated, non-contingent exposure. Specifically, in the CPP paradigm, the conditioned reinforcing effects of cocaine, assessed by an animal's preference for a cocaine-, relative to saline-, associated context, are intensified in animals with prior, repeated exposure to cocaine (e.g. Lett, 1989; Shippenberg et al., 1996).

Researchers have demonstrated cross-sensitization between the effects of psychostimulant drugs, other drug classes (e.g. opiates), and various stressors (Kalivas and Stewart, 1991; Steketee and Kalivas, 2011). In the case of cocaine, repeated exposure to the

drug sensitizes both the locomotor-activating and reinforcing effects of amphetamine, and vice versa (Bonate et al., 1997; Itzhak, 1997; Horger et al., 1992; Liu et al., 2007). The behavioural effects of many stressors have also been shown to cross-sensitize to cocaine. Indeed, repeated, intermittent exposure to social defeat (Covington and Miczek, 2001; Cruz et al., 2011), restraint (Araujo et al., 2003; Lepsch et al., 2005), variable stress (Lepsch et al., 2005; Prasad et al., 1998), and maternal separation (Kikusui et al., 2005), increases the locomotor response to a subsequent cocaine challenge. The converse is also true; animals given repeated, intermittent injections of cocaine show augmented behavioural responses to stressors such as footshock (Caggiula et al., 1998) and restraint (Maeda et al., 2009). Similar cross-sensitizing effects have been found in animals pre-exposed to other drugs, including amphetamine, morphine, and nicotine (e.g. Antelman et al., 1980; Deroche et al., 1995; Xu et al., 2004; Kita et al., 1999; Yu et al., 2010).

Given that many behavioural effects of stress and cocaine cross-sensitize, and that both types of stimuli activate the HPA axis (Munck and Guyre, 1986; Rivier and Vale, 1987), researchers initially speculated on a role for HPA signaling in cocaine-induced behavioural sensitization. However, in one study by Prasad and colleagues (1996), adrenalectomized rats repeatedly exposed to cocaine showed robust, unaltered sensitization of their locomotor response to a cocaine challenge 12 days after the pre-exposure regimen, suggesting that the development and long-term expression of cocaine-induced behavioural sensitization occurs independent of the HPA axis. Moreover, in another study, a cocaine pre-exposure regimen sufficient to sensitize cocaine-induced locomotion was without effect on cocaine-induced increases in plasma ACTH and corticosterone levels (Levy et al., 1992). To date, the requirement of HPA signaling in the cross-sensitizing effects of cocaine and stressors has not been studied; however, a critical role for this system would not be expected based on its lack of involvement in the expression of cocaine sensitization and in the long-lasting effects of cocaine

on other stress-induced behaviours, including footshock-induced reinstatement of cocaine seeking following prolonged drug-free periods (Erb et al., 1998).

Although evidence to date suggests that the HPA axis does not mediate cocaine-induced behavioural sensitization, CRF transmission does play an important role. Indeed, Przegaliński and colleagues (2005) demonstrated that systemic administration of the CRF_1 receptor antagonist, CP-154,526, blocked the sensitized locomotor response to a cocaine challenge given 5 days after repeated exposure to cocaine. Similarly, i.c.v. pretreatment with the CRF receptor antagonist, D-Phe $CRF_{12–41}$, attenuated the expression of locomotor sensitization when rats were given a cocaine challenge 28 days after 3 cocaine exposures (Erb and Brown, 2006). In addition, acute (Erb and Brown, 2006) or repeated, intermittent exposure to cocaine (Erb et al., 2003; 2005) produced an augmented locomotor response to i.c.v. injections of CRF given after drug-free periods of up to 4 weeks. Together, these findings support the notion that short-term exposure to cocaine can induce long-lasting changes in the function and responsivity of central CRF systems.

Evidence points to an important role for CRF signaling within the amygdala, specifically the CeA, in cocaine-induced behavioural sensitization. Using *in vivo* microdialysis in anesthetized rats, Richter and colleagues (1995) initially showed that extracellular CRF released in the CeA in response to a cocaine challenge is potentiated in rats with a history of cocaine, relative to saline, exposure. In another study, i.c.v. injection of CRF 12 days after repeated exposure to cocaine enhanced the expression of c-fos mRNA, a marker of neuronal activation (Curran and Morgan, 1995), in the CeA of cocaine, but not saline, pre-exposed animals (Erb et al., 2005). Consistent with these findings, cocaine pre-exposure sensitized footshock-induced expression of c-fos mRNA within the CeA, when footshock was administered up to 3 weeks following cocaine exposure in the previously drug-associated environment (Erb et al., 2004). Furthermore, recent evidence has shown that mice previously exposed to cocaine exhibit

potentiated CRF mRNA expression in the amygdala in response to forced swim stress after a 2-week drug-free period (Cleck et al., 2008). Although correlative in nature, these observations suggest that CRF signaling within the CeA, shown to be hyper-responsive in animals pre-exposed to cocaine, may be functionally important in the mediation of cocaine-induced behavioural sensitization.

Neuroplasticity within DAergic and glutamatergic interconnections of the VTA and terminal regions of the mesocorticolimbic DA system is considered responsible for the manifestation of psychostimulant sensitization (Vanderschuren and Kalivas, 2000; Steketee and Kalivas, 2011). In particular, repeated cocaine exposure produces long-lasting enhancements of cocaine-induced glutamate and DA release in the VTA and NAc that are associated with increased behavioural responsivity to the drug (Wise and Bozarth, 1987; Stewart, 1992; Steketee and Kalivas, 2011). As such, it is possible that CRF, which has been shown to regulate glutamate and DA transmission within the VTA and NAc in a manner that is modified by cocaine exposure, acts within these regions to mediate its effects on behavioural sensitization; in fact, several lines of evidence support this hypothesis. First, neurons in both the VTA and NAc express CRF receptors (Van Pett et al., 2000; De Souza et al., 1985) that, when activated, increase locomotor activity (Kalivas et al., 1987; Holahan et al. 1997). Second, CRF potentiates NMDA receptor-mediated excitatory transmission in VTA DA neurons (Ungless et al., 2003; Hahn et al., 2009), and increases their firing rate (Wanat et al., 2008). Conversely, CRF_1 receptor antagonists reduce cocaine-induced VTA DA neuron activity and DA release in the NAc and VTA (Lodge and Grace, 2005; Lu et al., 2003). Finally, CRF regulation of glutamate and DA signaling in the NAc and VTA is modified by repeated exposure to cocaine. For example, mice with a history of repeated cocaine exposure, relative to drug-naïve mice, show an enhanced CRF-induced potentiation of NMDA receptor-mediated transmission, and a novel CRF-induced potentiation of AMPA receptor-mediated transmission in VTA DA neurons (Hahn

et al., 2009). Moreover, intra-VTA injections of CRF selectively increase local glutamate and DA release (Wang et al., 2005a; 2007), as well as glutamate release within the NAc (Wang et al., 2002), in cocaine-experienced animals after drug-free periods of up to 3 weeks.

Considerable anatomical and functional evidence also indicates that amygdalar CRF may interact with the mesolimbic DA system to regulate cocaine-induced behavioural sensitization. First, DAergic projections from the VTA make direct synaptic contact with CRF neurons in the CeA (Eliava et al., 2003). DA transmission within the CeA positively regulates the expression of CRF mRNA (Day et al., 2002) and peptide (Stewart et al., 2008). Given that VTA DA neurons are innervated by CRF neurons from the CeA (Rodaros et al., 2007), it is possible that DAergic transmission in the CeA drives CRF release in the VTA, thereby facilitating behavioural sensitization. Consistent with this view, DA signaling within the amygdala has been critically implicated in psychostimulant sensitization. Specifically, repeated, intermittent exposure to amphetamine potentiates amygdalar DA release and locomotion in response to a low-dose amphetamine challenge after drug-free periods of up to 2 weeks (Harmer et al., 1997). Furthermore, bilateral 6-OHDA lesions of DA terminals in the amygdala prevent the development of behavioral sensitization induced by systemic or intra-VTA injections of amphetamine (Bjijou et al., 2002).

The overall activation of CeA efferents to the VTA, irrespective of their CRF content, may also play an important role in regulating the expression of behavioural sensitization. Activity within the CeA has been shown to positively regulate DA release in the NAc (Phillips et al., 2003), presumably via direct excitation (Rodaros et al., 2007; Tagliaferro and Morales, 2008) or polysynaptic disinhibition (Wallace et al., 1992) of VTA DA neurons. As such, hyperactivation of the CeA seen in cocaine-sensitized animals in response to stress and CRF treatment (Erb et al., 2004; 2005) may facilitate DAergic transmission within the NAc, and thereby promote the expression of behavioural sensitization.

1.3.3. CRF and Cocaine Withdrawal-induced Anxiety

Cessation of chronic cocaine use is commonly associated with states of negative mood, anhedonia, agitation, and heightened anxiety, particularly during the initial withdrawal, or so called "crash", period (Gawin and Kleber, 1986). Severe anxiety during withdrawal is considered one of the most important factors maintaining repetitive cycles of cocaine abuse (Gawin and Ellinwood, 1989). Using well-validated behavioural models of anxiety, researchers have demonstrated heightened levels of anxiety in laboratory rats and mice during the acute phase of cocaine withdrawal. Specifically, withdrawal-induced anxiety has been demonstrated 24-48 h after repeated daily cocaine injections using the defensive burying task (Harris and Aston-Jones, 1993; Basso et al., 1999), EPM (Sarnyai et al., 1995; DeVries et al., 1998; Rudoy and Van Bockstaele, 2007; Perrine et al., 2008; Hall et al., 2010), light-dark transition task (Costall et al., 1989), open field task (Yang et al., 1992), and acoustic startle paradigm (Gordon and Rosen, 1999; Bijlsma et al., 2010). Each of these behavioural measures of anxiety relies on the animal either choosing between areas of an apparatus that are ostensibly more or less anxiety-provoking (i.e., EPM, light-dark transition, open field), or responding to a discrete and anxiety-provoking stimulus (defensive burying, acoustic startle).

Although heightened states of behavioural anxiety have been reported beyond the first 48 h after withdrawal from cocaine, the expression of anxiety at these times is generally only revealed under conditions in which an acute aversive stimulus is presented at the time of testing (e.g. a shock from an electrified probe in the defensive burying procedure, or a footshock-associated tone in the acoustic startle paradigm), or the subject is re-exposed to contextual cues previously associated with cocaine administration just prior to testing. For example, Erb and colleagues (2006b) showed that rats tested in the EPM and light-dark transition tasks 10 days after cessation of cocaine injections showed heightened levels of anxiety, but only if they were returned to a previously cocaine-paired environment just before testing. In another study, rats

trained to self-administer cocaine in 6-h daily sessions demonstrated potentiated shock-induced anxiety in the defensive burying task up to 6 weeks after termination of cocaine self-administration (Aujla et al., 2008). In contrast, Mantsch and colleagues (2008) demonstrated that rats trained to self-administer cocaine under similar conditions failed to show heightened levels of anxiety 2-3 weeks after termination of drug self-administration, when testing occurred under baseline conditions in the EPM, light-dark transition, and open field tasks.

In contrast to studies of cocaine-induced behavioural sensitization, relatively little research has been done to explore the role of the HPA axis in the expression of cocaine withdrawal-induced anxiety. Several reports do reveal, however, that indices of HPA activity are increased in animals undergoing acute withdrawal from chronic cocaine exposure. For example, rats exposed to a 14-day "binge" pattern of cocaine administration (e.g. multiple, clustered, daily injections) showed significantly elevated plasma ACTH and corticosterone levels at 1 and 2, but not 10 days of withdrawal (Zhou et al., 2003a; b; Alves et al., 2008; but see Zhou et al., 2011). Interestingly, this same schedule of cocaine administration failed to alter hypothalamic CRF mRNA expression at any point during cocaine withdrawal (Zhou et al., 1996; 2003a; b). In addition, many studies using a less intense cocaine regimen (e.g. 7-14 single, daily injections) report no change in plasma corticosterone levels during the period of acute withdrawal associated with pronounced expression of anxiety (Avila et al., 2003; Baumann et al., 2004; Mantsch et al., 2007; but see Avila et al., 2004). Taken together, these results indicate that the HPA axis is activated during acute (24-48 h) withdrawal from cocaine in a dose- and schedule-dependent manner, but the importance of this activation for the expression of behavioural anxiety is unclear.

Several studies to date have directly implicated central CRF signaling in the elevated anxiety state seen during the first 48 h of cocaine withdrawal. For example, i.c.v. treatments with a CRF antiserum before daily cocaine exposures (Sarnyai et al., 1995), or i.c.v. injections

of the CRF receptor antagonist, α-helical CRF_{9-41}, at the time of testing (DeVries and Pert, 1998), block the expression of behavioural anxiety in the EPM 24-48 h after withdrawal from cocaine. Similarly, 48 h after withdrawal from cocaine (relative to saline), increases in defensive burying behaviours are blocked by i.c.v. pretreatment with the CRF receptor antagonist, D-Phe CRF_{12-41} (Basso et al., 1999).

Considerable evidence suggests that CRF acts within the amygdala, particularly the CeA, to mediate the expression of anxiety after withdrawal from cocaine. Indeed, the elevated levels of anxiety observed during the early period of withdrawal from cocaine (first 24-48 h) are associated with heightened basal levels of amygdalar CRF transmission. For example, immunoreactive CRF content in the amygdala is reduced 48 h after withdrawal from repeated cocaine exposure, suggesting increased release of the peptide (Sarnyai et al., 1995). Similar reductions in CRF-like immunoreactivity in the amygdala (Zorrilla et al., 2001), and increases in dialysate CRF concentrations in the CeA (Richter and Weiss, 1999), are seen in the 12 h following a 12-h cocaine self-administration session; together, these results are also indicative of increased peptide release. Accordingly, decreased CRF receptor binding in the amygdala (Ambrosio et al., 1997), and increased CRF mRNA expression in the amygdala (Zhou et al., 2003b; 2010), and CeA specifically (Erb et al., 2004; Maj et al., 2003), have been observed within the first 48 h of cocaine withdrawal.

Studies concerning a role for CeA CRF in anxiety states associated with withdrawal from cocaine have been extended to withdrawal from ethanol and opiates. These studies have yielded findings largely consistent with the cocaine work. Specifically, intra-CeA injections of the CRF and CRF_1 receptor antagonists, α-helical CRF_{9-41} and antalarmin, reverse the expression of ethanol withdrawal-induced anxiety (Rassnick et al., 1993) and morphine withdrawal-induced place aversion (Heinrichs et al., 1995), respectively.

1.3.4. CRF and Cocaine-related Behaviour Summary

Altogether, the experimental findings reviewed in the preceding sections represent our current understanding of how central CRF transmission regulates different cocaine-related behaviours. Exposure to acute stress reinstates extinguished cocaine-seeking behaviour, and does so via functional alterations in CRF transmission that occur as a consequence of prior cocaine experience. Likewise, changes in central CRF transmission underlie the expression of cocaine-induced behavioral sensitization, and mediate the expression of behavioural anxiety after withdrawal from cocaine.

Given the established role for CRF in the regulation of cocaine-related behaviours, it is important to investigate other neurochemical systems with the capacity to interact, and particularly interfere, with CRF to mediate its behavioural effects. As mentioned, the experiments presented in this dissertation were designed to explore the interaction between CRF and two other systems importantly related to the central regulation of stress responses in the expression of cocaine-related behaviours. These systems, which will be described in the next sections, are TCAP and the eCBs.

1.4. Teneurin C-terminal Associated Peptides

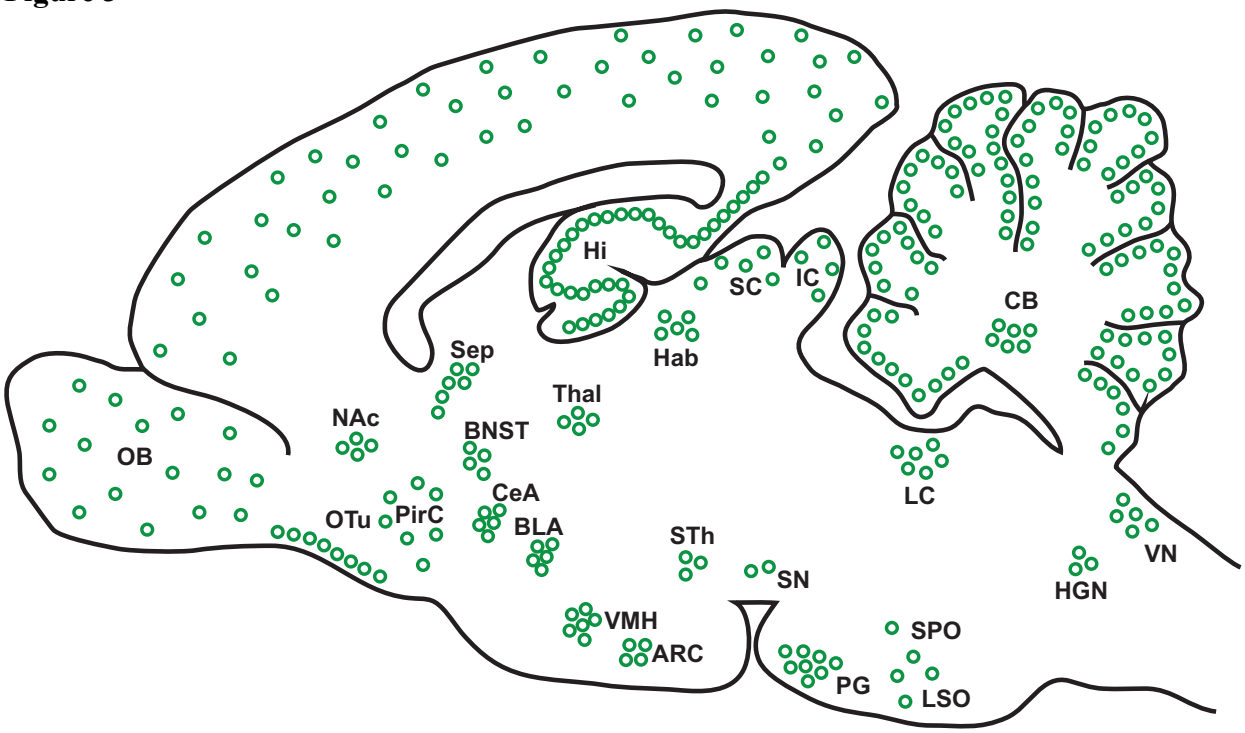
The TCAP family is a recently discovered family of endogenous neuropeptides (Qian et al., 2004; Wang et al., 2005b). It consists of four highly conserved, 40- or 41-amino acid bioactive peptides (TCAP-1-4), each found at the carboxy terminus of one of the four teneurin transmembrane proteins (Tucker and Chiquet-Ehrismann, 2006). TCAP-3, the first member of the family to be characterized, was discovered by Dr. David Lovejoy and colleagues during a low-stringency screen of a rainbow trout cDNA library for peptides with sequence similarity to the CRF family (Qian et al., 2004). In fact, the primary structures of all four TCAP sequences share about 20% sequence identity with CRF (Lovejoy et al., 2006). The TCAP family is also

strongly conserved across species, with homologues present in invertebrates (e.g. drosophila) and vertebrates (e.g. rats, humans) (Lovejoy et al., 2006). Each TCAP sequence possesses numerous cleavage motifs, indicating that the bioactive peptide may be enzymatically cleaved from the extracellular region of the teneurin transmembrane protein (Qian et al., 2004; Lovejoy et al., 2006). In addition, TCAP-1 and -3 mRNA can be transcribed and translated independently of the teneurin (Chand et al., unpublished). To date, none of the TCAP peptides have been purified and, thus, the molecular forms of the circulating peptides are unknown.

TCAP-1 is widely expressed in the adult rat brain, including within forebrain and limbic regions critical for the regulation of stress and anxiety (Wang et al., 2005b). Specifically, TCAP-1 mRNA and protein are highly expressed in the hippocampus, CeA, BLA, ventromedial nucleus of the hypothalamus, and piriform cortex, whereas lower levels are found in the BNST, cerebellum, and brainstem (see Figure 3; Wang et al., 2005b; Torres et al., 2011; Chand et al., unpublished). Notably, TCAP-1 is strongly expressed in brain regions rich in CRF neurons and receptors (Aguilera et al., 2004). TCAP-1 mRNA and protein also show similar, although more widespread, patterns of central expression to those of teneurin-1 (Li et al., 2006; Tucker et al., 2007; Kenzelmann et al., 2008). At the cellular level, teneurin-1 immunoreactivity is localized to the plasma membrane, whereas TCAP-1 immunoreactivity is more uniformly expressed throughout the cytosol (Chand et al., 2011; Torres et al., 2011), providing support for the notion that TCAP-1 acts in a manner distinct from its associated teneurin protein.

Studies using exogenous treatment with a well-characterized, synthetic form of TCAP-1 have revealed that TCAP-1 is internalized into neurons via calveoli-mediated endocytosis following a putative interaction with the teneurin protein, or with the transmembrane glycoprotein, β-dystroglycan (β-DG; Chand et al., 2011). Accordingly, strong binding of fluorescently-labeled TCAP-1 has been shown within the hippocampus (Chand et al., 2011), a region that densely expresses both teneurin-1 and β-DG (Li et al., 2006; Zaccaria et al., 2001).

Figure 3



Schematic of the distribution of TCAP-1 mRNA and protein in the rodent brain. Abbreviations: arcuate nucleus of the hypothalamus (ARC), basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), cerebellum (CB), habenula (Hab), hippocampus (Hi), hypoglossal nucleus (HN), inferior colliculus (IC), lateral superior olive (LSO), locus coeruleus (LC), nucleus accumbens (NAc), olfactory bulb (OB), olfactory tubercle (OTu), piriform cortex (PirC), pontine gray (PG), septum (Sep), substantia nigra (SN), subthalamic nucleus (STh), superior colliculus (SC), superior olive (SPO), thalamus (Thal), ventral tegmental area (VTA), ventromedial nucleus of the thalamus (VMH). Adapted from Tan et al., unpublished.

Moreover, both teneurin-1 and β-DG are internalized into cultured immortalized neurons following TCAP-1 treatment (Chand et al., 2011).

Synthetic TCAP-1 treatment induces a number of *in vitro* effects on neuronal signaling and structure. For example, exogenous TCAP-1 recruits a number of intracellular systems, including cAMP (Wang et al., 2005b), cAMP response element-binding protein (CREB), activating transcription factor-1 (ATF1), mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinase (ERK), ribosomal S6 kinase (p90RSK), Filamin A, and stathmin (Chand et al., 2011). In fact, TCAP-1 treatment modulates cAMP accumulation and cell proliferation in a comparable manner, increasing and decreasing both at low and high concentrations, respectively, in immortalized neuron cultures (Wang et al., 2005b). TCAP-1 application also upregulates cytoskeletal protein expression (e.g. β-actin, β-tubulin) and increases neurite and axonal outgrowth in cultures of immortalized mouse hypothalamic and primary hippocampal neurons (Al Chawaf et al., 2007a). ERK-p90RSK-mediated activation of Filamin A and ERK-mediated activation of stathmin are believed to underlie the pronounced reorganization of actin and tubulin cytoskeletons, respectively, seen in response to TCAP-1 treatment (Chand et al., 2011). These actions of exogenous TCAP-1 resemble those of teneurin proteins themselves, which have been implicated in transcriptional regulation (Bagutti et al., 2003; Nunes et al., 2005), neurite outgrowth (Rubin et al., 1999), axon guidance (Young and Leamey, 2009), cell adhesion (Rubin et al., 2002; Leamey et al, 2008), and cell proliferation (Kinel-Tahan et al., 2007).

In vitro TCAP-1 treatment also induces a variety of neuroprotective effects. For example, TCAP-1 decreased necrosis and increased proliferation of cultured hypothalamic neurons undergoing alkaloid pH stress; this occurred via an upregulation of reactive oxygen species scavenging systems (Trubiani et al., 2007). Furthermore, cultured neurons treated with TCAP-1, relative to vehicle, exhibit higher survivability under conditions of high confluency

(Wang and Lovejoy, unpublished), high calcium concentrations (Lovejoy et al., unpublished), and hypoxic stress (Trubiani and Lovejoy, 2008; Ng et al., unpublished). Interestingly, TCAP-1 has been shown to modulate translation of brain-derived neurotrophic factor (BDNF), a well-known mediator of structural plasticity and neuroprotection (Cowansage et al., 2010; Marini et al., 2008; Lipsky and Marini, 2007), in immortalized hypothalamic neurons (Ng et al., 2010). More recent evidence suggests that the structural and neuroprotective effects of TCAP-1 may reflect a more fundamental role for TCAP-1 in cellular energy regulation. Specifically, acute treatment of cultured immortalized neurons with TCAP-1 caused a significant decrease in intracellular concentrations of adenosine triphosphate (ATP; Xu et al., 2011). In contrast, chronic TCAP-1 treatment increased the presence of ATP, decreased concentrations of lactic acid, and increased intracellular glucose (Xu et al., 2011). Taken together, these findings suggest that TCAP-1 plays a regulatory role in neuron signaling and structure, as well as in cell survivability under conditions of stress.

1.4.1. TCAP-1 and Stress-related Behaviour

Given the expression of TCAP-1 in limbic brain regions that mediate, or are particularly sensitive to, the effects of stress (e.g. amygdala, hippocampus, hypothalamus), it has been hypothesized that the stress-regulating actions of TCAP-1 seen *in vitro* manifest in the regulation of stress-related behaviours. Indeed, several studies to date demonstrate that TCAP-1 treatment modulates the expression of emotional behaviours. The first such study was conducted by Wang and colleagues (2005b), who demonstrated that acute TCAP-1 administration into the BLA reduced the startle response of rats that displayed high emotionality prior to test (i.e. above average baseline startle amplitude), and enhanced the startle response of those that displayed low baseline emotionality. In the same report, repeated (5-day) i.c.v. treatment with TCAP-1, relative to saline, reduced the amplitude of acoustic startle in rats when

tested 5 days and 3 weeks after TCAP-1 exposure (Wang et al., 2005b). These results, consistent with a known role for TCAP-1 in the structural plasticity of neurons (Al Chawaf et al., 2007a; Tan et al., 2011), were the first to demonstrate that TCAP-1 can induce long-term changes in stress-related behaviour. Similarly, in the EPM, acute i.c.v. treatment with TCAP-1, relative to saline, increased stretch-attend postures (a type of risk-assessment behaviour [Kaesermann, 1986]) (Tan et al., 2009), whereas rats exposed for 10 days to i.c.v. TCAP-1 showed increased open arm time and decreased closed arm entries when tested 24 h after the final TCAP-1 treatment (Tan et al., 2011).

TCAP-1 has also been found to modulate several behavioural effects of CRF in a primarily, though not exclusively, inhibitory manner. For example, in a study by Al Chawaf and colleagues (2007b), 5 days of i.v. TCAP-1 injections decreased the number of stretch-attend behaviours made by rats in the EPM in response to an i.c.v. CRF challenge given 5 days after TCAP-1 treatment. Furthermore, 10 days after this same treatment, rats pre-exposed to TCAP-1 failed to show reductions in center time and entries in the open field task in response to i.c.v. CRF (Al Chawaf et al., 2007b). However, in the same study, TCAP-1 pre-exposure potentiated the anxiety-like effects of a CRF challenge in the open field when CRF was given intravenously (Al Chawaf et al., 2007b). Moreover, 5 days of i.c.v. TCAP-1 injections increased CRF-induced anxiety in the EPM and open field, but blocked CRF-induced increases in acoustic startle responses up to 3 weeks after initial TCAP-1 exposure (Tan et al., 2008). Collectively, these results point to a complex interaction between TCAP-1 and CRF that depends upon a variety of factors, including the route and regimen of TCAP-1 and CRF treatment, the behavioural test, and presumably the baseline reactivity of the rats being tested. In general, the modulatory actions of TCAP-1 on stress-related behaviours are more robust when assessed using homogeneous samples of subjects under stressful (i.e. CRF challenge), relative to baseline, conditions.

The brain loci in which TCAP-1 acts to modulate CRF-induced behaviour, and the mechanism of its action, are not known. However, the central distributions of TCAP-1, as well as teneurin and β-DG, two proteins believed to serve as binding sites for TCAP-1, have been shown to be widespread, and particularly pronounced in limbic and frontal brain regions (Wang et al., 2005b; Torres et al., 2011; Li et al., 2006; Kenzelmann et al., 2008; Zaccaria et al., 2001; Ohysuka-Tsurumi et al., 2004). To date, strong binding of fluorescently-labeled TCAP-1 has been shown within the hippocampus (Chand et al., 2011); binding within other brain regions has yet to be characterized. Accordingly, 10-day i.c.v. treatment with TCAP-1 sufficient to reduce anxiety-like behaviour in the EPM increased spine density in the CA1 and CA3 regions of the hippocampus, and decreased branching in CA3 basilar dendrites (Tan et al., 2011). Moreover, acute i.c.v. injections of TCAP-1 blocked CRF-induced expression of c-fos in the CA1, CA2, CA3, dentate gyrus, and dorsal raphe, and attenuated the CRF effect in the CeA, BLA and medial PFC (mPFC; Tan et al., 2009). *In vitro* evidence suggests that TCAP-1 exerts these and other effects independent of CRF receptor transmission. Specifically, Wang and colleagues (2005b) found that TCAP-1 treatment stimulated cAMP accumulation in CRF_1 receptor-expressing immortalized neurons, irrespective of pretreatment with the selective CRF_1 receptor antagonist, PD171729. Moreover, TCAP-1 modified cAMP levels in cells that express neither CRF_1 nor CRF_2 receptors (Wang et al., 2005b). TCAP-1 has also been shown to act independently of CRF receptors to activate CREB and ATF1, and downregulate activator protein-1 (AP-1; De Almeida et al., 2011; Nock et al., unpublished). Furthermore, TCAP-1 is without effect on basal or CRF-induced plasma corticosterone levels, suggesting that the actions of TCAP-1 are CRF_1 receptor- and HPA-independent (Al Chawaf et al., 2007b). Given that TCAP-1 is expressed in CRF-rich brain regions (Wang et al., 2005b; Aguilera et al., 2004), modulates signal transduction cascades recruited by CRF (e.g. cAMP, CREB, ERK; Grammatopoulos and Chrousos, 2002; Stern et al., 2011), and induces changes in neuronal

structure and connectivity (Al Chawaf et al., 2007a; Tan et al., 2011), it is possible TCAP-1 may act downstream of CRF receptor activation to modify cell signaling and structure in a manner that serves to inhibit certain cellular and behavioural effects of CRF.

Collectively, these findings describe an important regulatory role for TCAP-1 in neuronal and behavioural responses to stress and CRF. No studies to date have investigated such a role for TCAP-1 in subjects with prior cocaine experience. In fact, experiments comprising the present dissertation are the first to characterize the effects of TCAP-1 on cocaine-related behaviours induced, or mediated, by CRF. Before detailing the objectives of this work, consideration will be given to the second stress-related neurochemical system examined in this dissertation - the eCBs - and its interactions with CRF in the central regulation of stress- and cocaine-related behaviour.

1.5. Endocannabinoids

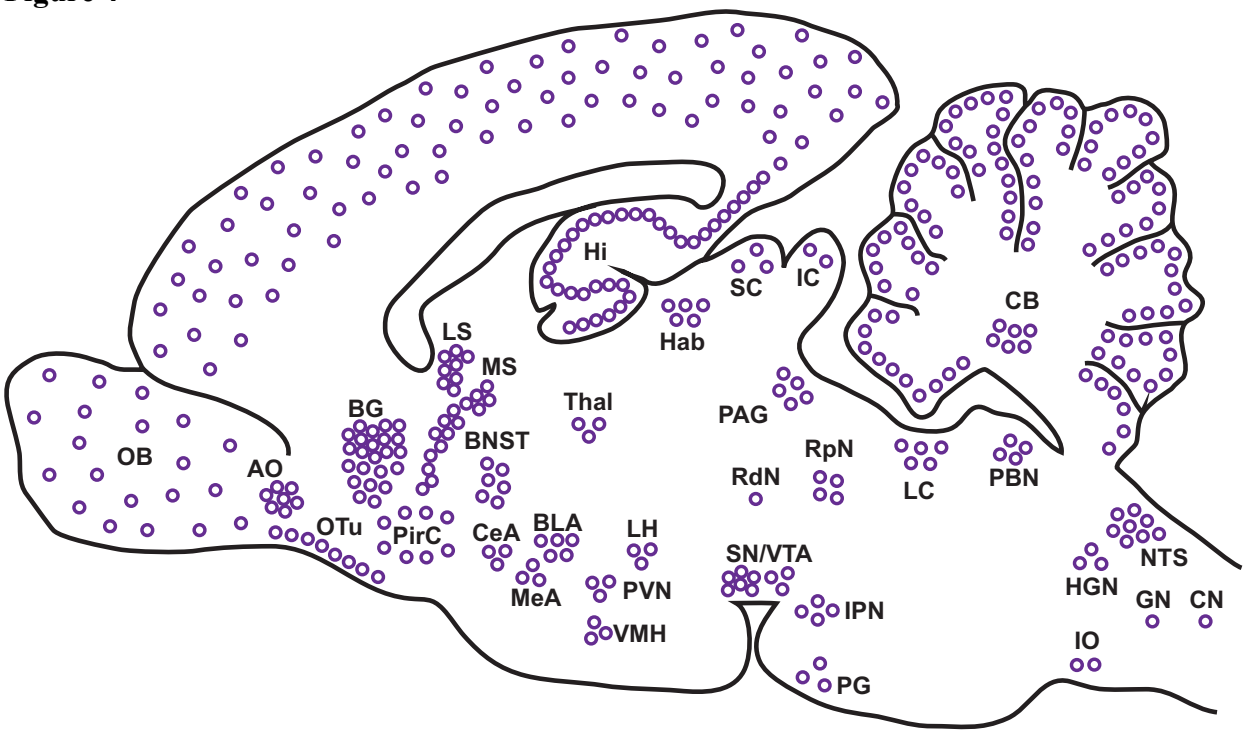
The endocannabinoids (eCBs) comprise a family of bioactive lipid metabolites that act as retrograde signaling molecules in both the central and peripheral nervous systems (Lovinger, 2008). Following activation of a postsynaptic neuron, eCBs such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesized 'on demand' from phospholipids within the postsynaptic membrane, and act presynaptically to suppress the release of a variety of neurotransmitters and neuromodulators (Szabo and Schlicker, 2005). This suppression is mediated predominantly through presynaptically expressed cannabinoid-1 (CB_1) receptors. Coupled to $G_{i/o}$ proteins, CB_1 receptors act to inhibit adenylate cyclase activity, activate potassium channels, and inhibit voltage-gated calcium channels, thereby reducing the excitability of the presynaptic neuron and inhibiting neurotransmitter release (Howlett and Fleming 1984; Howlett 1987; Mackie and Hille 1992; Mackie et al. 1995; Twitchell et al. 1997; Freund et al., 2003). Central CB_1 receptor expression is such that eCBs are capable of

suppressing the release of a diverse set of neurochemicals, including GABA, glutamate, acetylcholine, noradrenaline, and serotonin (Nakazi et al., 2000; Ohno-Shosaku et al., 2001; Schlicker and Kathman, 2001). Moreover, CB_1 receptors are considered among the most widely distributed and abundant G protein-coupled receptors in the brain, with notable expression in the piriform, prefrontal and cerebellar cortices, as well as the hippocampus, amygdala, striatum, and hypothalamus (See Figure 4; Herkenham et al., 1990; 1991; Tsou et al., 1998; Moldrich and Wenger, 2000). Particularly high density is observed in cortical and limbic regions associated with emotional responses (Viveros, 2007). Accordingly, *in situ* hybridization studies reveal significant colocalization between the mRNA of CB_1 receptors and both CRF peptide and CRF_1 receptors in numerous stress-related brain regions (Hermann and Lutz, 2005; Cota et al., 2007).

In recent years, eCBs have emerged as critical regulators of neuroendocrine responses to stress, primarily via their actions at presynaptic CB_1 receptors. In fact, considerable evidence indicates that eCB signaling negatively regulates activity of the HPA axis. The first such evidence came from Hillard and colleagues, who demonstrated that pharmacological inhibition of eCB uptake and degradation, and CB_1 receptor activation, blunted restraint stress-induced corticosterone secretion in mice (Patel et al., 2004). Furthermore, CB_1 receptor antagonism enhanced basal corticosterone levels, and potentiated the HPA axis response to restraint (Patel et al., 2004). Support for these findings came from numerous genetic and pharmacological studies demonstrating that CB_1 receptor activation serves to constrain basal and stress-induced HPA activity (Manzanares et al., 1999; Barna et al., 2004; Haller et al., 2004; Uriguen et al., 2004; Doyon et al., 2006; Wade et al., 2006; Cota et al., 2007; Aso et al., 2008; Steiner et al., 2008; Ginsberg et al., 2010).

Exposure to stress has been shown to critically modify eCB signaling in various brain regions, and these modifications have been implicated in the regulation of the HPA axis. For example, 30-min exposure to restraint stress caused reductions in tonic AEA content in the PFC,

Figure 4



Schematic of the distribution of CB_1 receptors in the rat brain.

Abbreviations: anterior olfactory nucleus (AO), basal ganglia (BG), basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST), cerebellum (CB), cuneate nucleus (CN), gracile nucleus (GN), habenula (HB), hippocampus (Hi), hypoglossal nucleus (HGN), inferior colliculus (IC), inferior olive (IO), interpeduncular nucleus (IPN), lateral hypothalamus (LH), lateral septum (LS), locus coeruleus (LC), medial septum (MS), medial nucleus of the amygdala (MeA), nucleus of the solitary tract (NTS), olfactory bulb (OB), olfactory tubercle (OTu), parabrachial nucleus (PBN), paraventricular nucleus of the hypothalamus (PVN), periaqueductal gray (PAG), piriform cortex (PirC), pontine gray (PG), raphe nucleus (RpN), red nucleus (RdN), substantia nigra (SN), superior colliculus (SC), thalamus (Thal), ventral tegmental area (VTA), ventromedial nucleus of the thalamus (VMH). Adapted from Herkenham et al. (1991).

hippocampus (Hill et al., 2007), and amygdala (Patel et al., 2005a; Rademacher et al., 2008; Hill et al., 2009). In contrast, restraint stress enhanced 2-AG content in the PFC, hypothalamus, and hippocampus (Hill et al., 2007; 2011; Evanson et al., 2010; Wang et al., 2011; see Hill and McEwen, 2010 for review). Recent evidence suggests that the reduction in amygdalar AEA content seen following stress serves to disinhibit the HPA axis. Hill and colleagues (2009) demonstrated that local administration of the CB_1 receptor agonist, HU210, or the AEA degradation inhibitor, URB597, into the BLA (but not the CeA) attenuated the corticosterone response to restraint stress (see also, Ganon-Elazar and Akirav, 2009). In contrast, intra-BLA injection of the CB_1 receptor antagonist, AM251, induced an overall increase in corticosterone secretion. Moreover, amygdalar AEA content in rats exposed to restraint was inversely correlated with serum corticosterone levels, suggesting that reductions in tonic AEA signaling may exert a permissive influence over HPA activation (Hill et al., 2009). On the other hand, the enhancements in 2-AG content seen in the PFC and PVN following restraint stress have been implicated in the termination of the HPA response. More specifically, stress-induced mobilization of prefrontal and hypothalamic 2-AG mediates the negative feedback induced by glucocorticoids, like corticosterone, on HPA activity. Indeed, pretreatment with the glucocorticoid receptor antagonist, RU-486, prevented the restraint-induced elevation in 2-AG content within the mPFC, and intra-PFC administration of the CB_1 receptor antagonist, AM251, potentiated corticosterone secretion following restraint (Hill et al., 2011). Similarly, intra-PVN infusion of AM251 blocked the suppressive effects of the synthetic glucocorticoid, dexamethasone, on restraint-induced ACTH and corticosterone secretion (Evanson et al., 2010). From this evidence, researchers have theorized a possible bidirectional role for eCB signaling in the regulation of the HPA axis; specifically, downregulation of tonic AEA signaling in response to stress may promote HPA activity, whereas stress-induced upregulation of 2-AG signaling may suppress HPA activity (see Hill and McEwen, 2010 for review).

1.5.1. Endocannabinoids and Stress-related Behaviour

eCB signaling also regulates numerous emotional behaviours, including anxiety. Studies using various preclinical models of anxiety-like behaviour have shown that pharmacological enhancement of CB_1 receptor transmission, using agonists or eCB uptake/degradation inhibitors, is generally anxiolytic (e.g. Patel and Hillard, 2006; Bortolato et al., 2006; Naidu et al., 2007; Rubino et al., 2007; but see Griebel et al., 2005). These CB_1-mediated effects tend, however, to be dose- and region-specific. Specifically, dose-response studies have demonstrated that the effect of direct CB_1 receptor stimulation appears to be biphasic, such that low doses of cannabinoids reduce anxiety, and high doses evoke anxiety (Moreira and Wotjak, 2010). Moreover, local injections of CB_1 receptor agonists or eCB uptake inhibitors into the PFC, ventral hippocampus, dorsolateral periaqueductal gray (PAG), and CeA reduce anxiety-like behavior in the EPM (Rubino et al., 2008a; b; Moreira et al., 2007; Zarrindast et al., 2008; but see Onaivi et al., 1995; Campos et al., 2010), whereas CB_1 receptor agonists injected into the dorsal hippocampus and BLA are anxiogenic (Roohbakhsh et al., 2007; Rubino et al., 2008a; but see Ganon-Elazar and Akirav, 2009). In contrast to systemic CB_1 receptor activation, systemic blockade of CB_1 receptors generally increases anxiety-like behaviour (e.g. Haller et al., 2004; Rodgers et al., 2005; but see Griebel et al., 2005; Lafenêtre et al., 2007). CB_1 receptor knockout mice similarly exhibit a heightened anxiety phenotype, as demonstrated using the EPM (Haller et al., 2002; 2004; Hill et al., 2011), light-dark box, and social interaction test (Martin et al., 2002).

Despite evidence of its anxiolytic effects, CB_1 receptor transmission has also been shown to mediate various behavioural effects of stress. For example, CB_1 receptor-deficient mice with a history of ethanol self-administration failed to show an increase in ethanol consumption following exposure to footshock stress, relative to their wild-type counterparts (Racz et al., 2003). Moreover, CB_1 receptor knockout mice showed no increase in the latency to

respond to a painful heat stimulus following exposure to forced swim stress, indicative of impaired stress-induced analgesia (Valverde et al., 2000). Pharmacological blockade of CB_1 receptors, either systemically or locally in the dorsolateral PAG or BLA (but not CeA), also prevented the analgesic response to footshock stress (Hohmann et al., 2005; Connell et al., 2006; Kurrikoff et al., 2008). In contrast, intra-PAG infusion of the 2-AG and AEA degradation inhibitors, URB602 and URB597, enhanced footshock stress-induced analgesia in a CB_1 receptor-dependent manner (Hohmann et al., 2005). Accordingly, footshock stress enhanced tissue levels of 2-AG and AEA in the dorsal midbrain (Hohmann et al., 2005). Taken together, these and other findings suggest that stress-induced mobilization of eCBs may mediate some effects of stress on behaviour (Hill and McEwen, 2010).

1.5.2. Endocannabinoids and Cocaine-related Behaviour

1.5.2.1. Endocannabinoids and Reinstatement of Cocaine Seeking

CB_1 receptor transmission has been reported to induce and mediate various cocaine-related behaviours, including the reinstatement of cocaine seeking. The first such report came from De Vries and colleagues (2001), who demonstrated that systemic treatment with the synthetic cannabinoid agonist, HU210, induced reinstatement of cocaine seeking in a CB_1 receptor-dependent manner. On the other hand, systemic pretreatment with the CB_1 receptor antagonist, SR141716, blocked the reinstatement induced by non-contingent exposure to cocaine or cocaine-associated cues (De Vries et al., 2001). Subsequent reports using this and more selective CB_1 receptor antagonists confirmed a role for CB_1 receptors in cocaine- and cue-induced reinstatement of cocaine seeking (Filip et al., 2006a; but see Ward et al., 2009). Striatal eCB signaling in particular has been implicated in this behaviour, as local infusions of the CB_1 receptor antagonist, AM251, into the NAc or dorsal striatum have been shown to attenuate cocaine seeking induced by a cocaine challenge (Xi et al., 2006).

It is worth noting that CB_1 receptor transmission does not mediate the effects of cocaine on the reinstatement of other extinguished drug-related behaviours, namely cocaine CPP. Recent work by Mantsch, Hillard and colleagues demonstrated that, following the development and extinction of cocaine CPP, systemic treatment with the selective CB_1 receptor agonist, CP-55,940, failed to increase the time spent by mice in the previously cocaine-paired environment (Vaughn et al., 2011). Further in contrast with previous reports using operant models of reinstatement (e.g. De Vries et al., 2001; Xi et al., 2006; but see Ward et al., 2009), the selective CB_1 receptor antagonist, AM251, was without effect on cocaine-induced reinstatement of extinguished CPP (Vaughn et al., 2011). These discrepant findings are likely due to differences in the modes of cocaine administration, the species used, and the behavioural outcomes measured in each study.

CB_1 receptor transmission has been shown to mediate reinstatement behaviour in animals trained to self-administer drugs of abuse other than cocaine. Indeed, treatment with CB_1 receptor agonists induces reinstatement of drug seeking in animals with self-administration histories of the agonist itself (Spano et al., 2004), as well as of heroin (De Vries et al., 2003), nicotine (Gamaleddin et al., 2011), or methamphetamine (Hiranita et al., 2008). CB_1 receptor antagonists also attenuate drug prime- and cue-induced reinstatement in animals trained to self-administer heroin (De Vries et al., 2003), nicotine (De Vries et al., 2005; Forget et al., 2009), ethanol (de Bruin et al., 2011; Cippitelli et al., 2005), methamphetamine (Anggadiredja et al., 2004), or various cannabinoid receptor agonists (Justinova et al., 2008). Interestingly, an isolated report demonstrated that systemic treatment with the AEA reuptake inhibitor, URB597, attenuated reinstatement of nicotine seeking induced by non-contingent nicotine or nicotine-associated cues (Forget et al., 2009). In general, pharmacological manipulations of eCB signaling using antagonists (e.g. De Vries et al., 2001; 2003; 2005) and inhibitors of eCB uptake and degradation (Cippitelli et al., 2008; Forget et al., 2009) are, themselves, without effect on

reinstatement behaviour.

To date, the role of eCB signaling in stress-induced reinstatement of drug seeking is unclear. In the original study demonstrating a definitive role for CB_1 receptor transmission in cocaine- and cue-induced reinstatement of cocaine seeking in rats, De Vries and colleagues (2001) reported that systemic treatment with the CB_1 receptor antagonist, SR141716, was without effect on footshock-induced reinstatement. Subsequent reports showed that ethanol-seeking behaviour induced by footshock stress was similarly unaffected by systemic administration of SR141716 (Economidou et al., 2006), or the AEA reuptake inhibitor, URB597 (Cippitelli et al., 2008). CB_1 receptor transmission has, however, been implicated in the effects of stress on the reinstatement of cocaine CPP. For example, systemic treatment with the selective CB_1 receptor antagonist, AM251, blocked the ability of forced swim stress to increase time spent by mice in the previously cocaine-paired environment (Vaughn et al., 2011). While, as mentioned, the many differences between operant and associative models of reinstatement likely contribute these discrepant findings, the evidence from Vaughn and colleagues (2011) support the notion that stress-induced cocaine seeking, like other behavioural effects of stress (e.g. Racz et al., 2003; Hohmann et al., 2005), can be mediated by CB_1 receptor transmission.

1.5.2.2. Endocannabinoids and Cocaine-induced Behavioural Sensitization

eCB signaling has also been implicated in the development and expression of cocaine sensitization, albeit with some inconsistency. Several groups have demonstrated that repeated, systemic pretreatment with the CB_1 receptor antagonist, SR141716, prior to daily cocaine injections is largely without effect on the sensitized locomotor response to a subsequent cocaine challenge (Lesscher et al., 2005; Gerdeman et al., 2008; Ramiro-Fuentes and Fernandez-Espejo, 2011). In contrast, Corbillé and colleagues (2007) reported that systemic pretreatment of mice with the CB_1 receptor antagonist, AM251, prior to a single cocaine injection prevented the sensitized locomotion seen 7 days later in response to a cocaine challenge (see also Thiemann et

al., 2008). Findings from studies using CB_1 receptor-deficient mice are similarly equivocal, reporting either intact (Martin et al., 2000) or impaired (Corbillé et al., 2007) development of cocaine sensitization in the genetically modified animals. The expression of cocaine sensitization, however, appears to be more consistently CB_1 receptor-mediated, given that acute SR141716 pretreatment prior to a cocaine challenge blocked the sensitized locomotor response seen in cocaine, relative to saline, pre-exposed animals (Filip et al., 2006a; Ramiro-Fuentes and Fernandez-Espejo, 2011). In general, the expression of behavioural sensitization to various drugs of abuse, including morphine, nicotine, and to a lesser extent, amphetamine, is more sensitive to manipulations of eCB signaling and CB_1 receptor transmission than the development of sensitization (Singh et al., 2004; Viganò et al., 2004; Kelsey and Calabro, 2008; Biala and Budzynska, 2010; Chiang and Chen, 2007; Thiemann et al., 2008). Despite considerable evidence of a role for eCBs in various behavioural effects of cocaine and stress, no work to date has explored their role in the cross-sensitization between cocaine and stress.

1.5.2.3. Endocannabinoids and Cocaine Withdrawal-induced Anxiety

A mediating role for eCBs has been established in the expression of many aversive effects of withdrawal from drugs of abuse. For example, CB_1 receptor-deficient mice with a prolonged history of ethanol self-administration failed to show any somatic signs of withdrawal 24 h following cessation of ethanol access, relative to their wild-type counterparts, despite showing normal ethanol responsivity (acute and chronic) and preference (Racz et al., 2003). Moreover, administration of the CB_1 receptor antagonist, SR141716, before testing in the EPM reversed the reduction in time spent in the open arms by rats withdrawn from chronic exposure to ethanol (Rubio et al., 2008; Onaivi, 2008) or diazepam (Onaivi, 2008). Similar anxiolytic effects of SR141716 have been observed in animals withdrawn from chronic cocaine exposure (Onaivi, 2008); however, this isolated report used an unspecified regimen of cocaine exposure,

and only a single dose of the CB_1 receptor antagonist. As such, the role of CB_1 receptor transmission in cocaine withdrawal-induced anxiety warrants more systematic investigation.

1.6. Thesis Rationale and Objectives

The preceding review of the published work to date provides substantial support for the notion that CRF signaling mediates numerous behavioural responses to stress, including those unique to subjects with prior exposure to drugs of abuse like cocaine. The review further points to an important modulatory role for the neuropeptide, TCAP-1, in stress-related and CRF-induced behaviours, and for the eCB system in numerous stress- and cocaine-related behaviours. An issue of particular relevance to the present dissertation, as yet unexplored, is the role of TCAP-1 in cocaine-related behaviours. Similarly, the role of the eCB system in the behavioral effects of stress in cocaine-experienced animals remains largely uncharacterized to date. Despite evidence of a neuroanatomical overlap between eCB and CRF systems, and a role for eCB signaling in the central regulation of the HPA axis, there is a paucity of data addressing a direct functional interaction between eCBs and CRF in the regulation of behavioural responses, including cocaine-related responses.

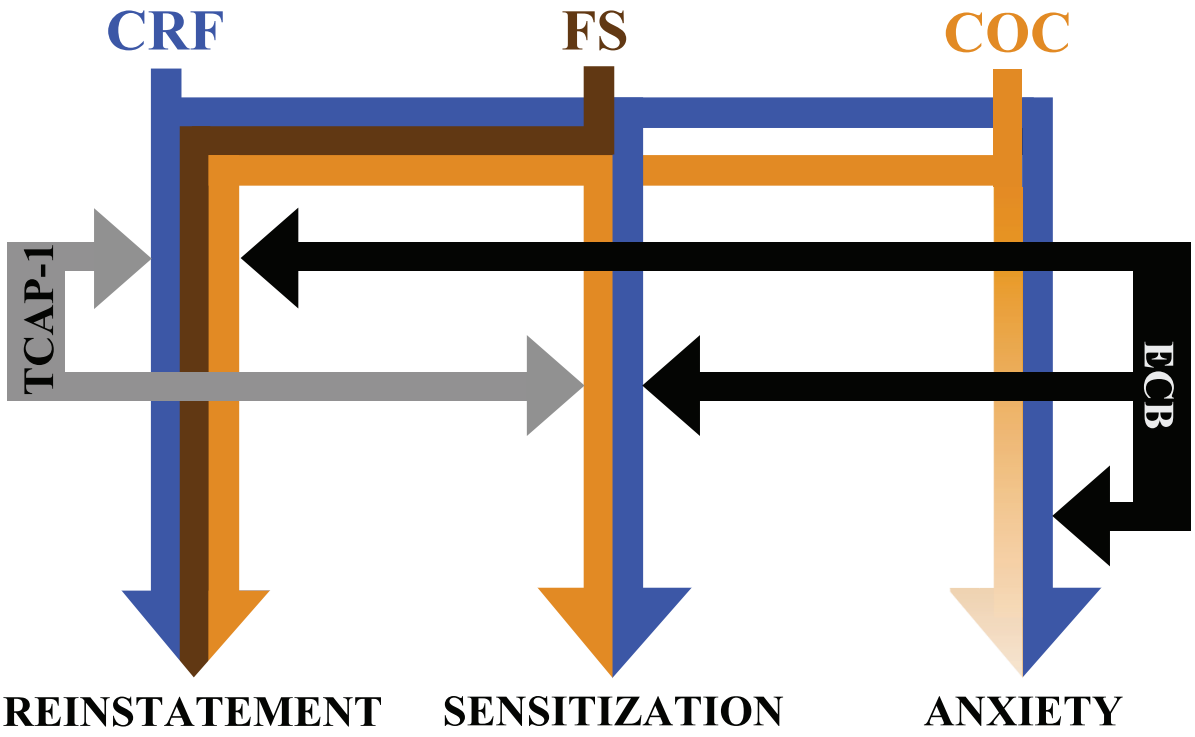
In addition to their ability to regulate stress-related behaviours, the TCAP-1 and eCB systems have other common characteristics. It was, in fact, a consideration of some of these similarities that prompted the parallel investigation of these two systems in the present dissertation. For example, TCAP-1 and CB_1 receptors exhibit pronounced regional overlap in their central expression. Indeed, both proteins are most densely expressed in the hippocampus, amygdala, cerebellum, hypothalamus and frontal cortex (Tsou et al., 1998; Torres et al., 2011), regions that also contain high levels of CRF receptors (Primus et al., 1997; Van Pett et al., 2000). In addition, the cellular actions of TCAP-1 and eCBs implicate many of the same intracellular systems, such as cAMP, CREB, MEK, and ERK (Wang et al., 2005b; Chand et al.,

2011; Isokawa, 2009; Davis et al., 2003; Derkinderen et al., 2003), all of which are also recruited by CRF receptor activation (Li et al., 1998; Kageyama et al., 2007; Gallagher et al., 2008). Finally, both systems have been found to modulate the activity of the neurotrophin, BDNF (Ng et al., 2010; Butovsky et al., 2005; Lemtiri-Chlieh and Levine, 2010; De Chiara et al., 2010), and to regulate neuronal survival under conditions of cellular stress, such as those induced by radical oxygen species (Trubiani et al., 2007; Marsicano et al., 2002; Comelli et al., 2010), hypoxia (Trubiani and Lovejoy, 2008; Ng et al., 2010; Nagayama et al., 1999), or excitotoxicity (Lovejoy et al., unpublished; Shen and Thayer, 1998; Hansen et al., 2002).

Extending on the noted similarities in some of the *in vitro* and *in vivo* effects of TCAP-1 and eCBs, the series of experiments presented in this dissertation constitute a systematic and parallel exploration of the modulatory actions of these two systems in cocaine- and anxiety-related behaviours. Specifically, the experiments were designed to explore whether TCAP-1 and eCBs interact with CRF to mediate its effects on the reinstatement of cocaine seeking, cocaine-sensitized locomotion, and anxiety-like behaviour. To this end, three main research objectives were developed to extend upon what is currently known about TCAP-1, eCB, and CRF signaling in stress- and cocaine-related neurophysiology and behaviour (see Figure 5 for schematic representation). The first objective (Chapter 3 experiments) was to determine whether administration of TCAP-1 modulates the effects of i.c.v. CRF on the reinstatement of cocaine seeking and cocaine-sensitized locomotion, and to compare these effects with those induced by footshock stress and/or a cocaine challenge. The second objective (Chapter 4 experiments) was to investigate whether administration of the CB_1 receptor antagonist, AM251, modulates the effects of i.c.v. CRF on the reinstatement of cocaine seeking and cocaine-sensitized locomotion, and to compare these effects with those induced by footshock stress and/or a cocaine challenge. The third and final objective (Chapter 5 experiments) was to determine whether AM251 administration modulates CRF- and cocaine withdrawal-induced

anxiety, and to explore whether any observed behavioral effects corresponded to changes in HPA axis activity.

Figure 5



Schematic depicting the experimental objectives of the dissertation.
Objective 1: to determine whether administration of TCAP-1 modulates CRF-, footshock (FS)-, or cocaine (COC)-induced reinstatement of cocaine seeking, and the expression of cocaine-induced behavioural sensitization to a CRF or cocaine challenge. Objective 2: to determine whether administration of the CB_1 receptor antagonist, AM251, modulates CRF-, footshock-, or cocaine-induced reinstatement of cocaine seeking, and the expression of cocaine-induced behavioural sensitization to a CRF or cocaine challenge. Objective 3: to determine whether administration of the AM251, modulates CRF- or cocaine withdrawal-induced anxiety and HPA axis activation.

CHAPTER 2

General Methods

CHAPTER 2

General Methods

2.1. Subjects

Male Long–Evans rats (Charles River, Montreal, QC; N=321; 250-275g initial weight) were used in Experiments 1, 2, 4, 5, 7, and 8. Male Wistar rats (Charles River; N=115; 250-275g) were used in Experiments 3 and 6. Rats were individually housed in plastic cages in a temperature- (21±1˚C) and humidity-controlled vivarium, and maintained on a reverse light schedule (lights on 1900–0700) with free access to water and standard laboratory rat chow. All procedures were performed in accordance with Canadian Council of Animal Care guidelines, and were approved by the University of Toronto animal care committee.

2.2. Surgery

2.2.1. Intracerebroventricular Cannulation

Under isoflurane anesthesia (3-5% in O_2; Benson Medical, Markham, ON, Canada), all rats were implanted with a 22-gauge cannula (Plastics One, Roanoke, VA, USA). The cannula was aimed 1 mm above the right lateral ventricle, according to the following stereotaxic coordinates: A/P: -1.0 mm from bregma; M/L: -1.4 mm from bregma; D/V: -2.7 mm from dura (Paxinos & Watson, 1997). The i.c.v. cannula was embedded in dental cement and anchored to the skull with jeweler's screws. At the end of the surgery, a stainless steel blocker extending 1 mm beyond the cannula tip was inserted into the cannula. Rats were given a 7-day recovery period before the start of any behavioural procedures. Cannula placements were verified by observing drinking behaviour following i.c.v. administration of angiotensin (100 μg/4μl; Sigma-Aldrich, Oakville, ON, Canada). Placements were considered accurate if the rat drank within 1 min of the injection, and sustained drinking for more than 2 min.

2.2.2. Intravenous Catheterization

Rats used in the drug self-administration experiments (Exp. 1, 2, 4) were implanted with a silastic i.v. catheter (Dow Corning, Midland, MI, USA; inner diameter: 0.51 mm; outer diameter: 0.94 mm) into the right jugular vein at the time of i.c.v. cannulation. The catheter was a total length of 12 cm, with 3 cm inserted into and secured to the vein with silk sutures. The remaining 9 cm was passed subcutaneously to the skull surface, where it exited into a modified 22-gauge cannula (Plastics One) that, along with the i.c.v. cannula, was embedded in dental cement and anchored to the skull with jeweler's screws. At the end of the surgery, the catheter was flushed with 0.2 ml of a solution containing 50% heparin (1000 IU/ml; Leo Pharma Inc., Thornhill, ON, Canada) and 50% dextrose (0.5 g/ml; Hospira, Montreal, QC, Canada), and a plastic blocker was placed over cannula to protect the catheter from external debris and maintain catheter patency. Rats were given a 7-day recovery period before the start of any behavioural procedures.

2.3. Drugs

Cocaine HCl (Medisca Pharmaceuticals, St. Laurent, QC, Canada) was dissolved in saline at concentrations of 3.5 mg/ml (injected in a volume of 65 µl; i.v.), or 10, 15, 20 and 30 mg/ml (injected in a volume of 1 ml/kg; i.p.). CRF (Sigma–Aldrich) was dissolved in saline (0.125 µg/µl), and injected in a volume of 4µl (0.5 µg; i.c.v.). Synthetic rat TCAP-1 (American Peptide Company, Sunnyvale, CA) was dissolved in saline (10^{-5}M, 10^{-4}M and $2x10^{-4}$M), and injected in a volume of 3µl (30, 300, 600 pmol, i.c.v.). AM251 [(N-(piperidin-1-yl)-5 (4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (Tocris Bioscience, Burlington, ON)] was dissolved in dimethyl-sulfoxide (DMSO; Sigma-Aldrich) at concentrations of 2.5, 25, and 50 µg/µl, and injected in a volume of 4 µl (10, 100, 200 µg; i.c.v.).

2.4. Microinjection Procedures

CRF, TCAP-1, and AM251 were infused (i.c.v.) using a 10 µl Hamilton syringe connected to a 28-gauge stainless steel injector (Plastics One) that extended 1 mm below the tip of the cannula into the lateral ventricle. Infusions took place over a 2-min period; injectors were left in place for an additional 2 min to prevent backflow.

2.5. Apparatuses

2.5.1. Drug Self-Administration Chambers

Drug self-administration chambers (Med Associates, St. Albans, VT, USA) were equipped with two retractable levers located 6 cm above a stainless steel rod floor. An infusion pump (Razel Scientific Instruments, Stamford, CN, USA) was activated by responses on one lever (active lever). Each active lever response was automatically recorded using a computer interface operating Med-IV software (Med Associates). Responses on the second lever (inactive lever) were recorded but did not result in activation of the pump. Each chamber was equipped with a white stimulus light, located directly above the active lever, and a white houselight. Each chamber was also fitted to deliver constant-current, intermittent, inescapable, electric footshock through a scrambler to the metal rod floor. Footshock was delivered according to a variable time schedule at a mean interval of 40 s (10-70 s range). Each shock (0.9 mA) was 0.5 s in duration.

2.5.2. Sucrose Self-Administration Chambers

Similar to the drug self-administration chambers, sucrose pellet self-administration chambers (Med Associates) were also equipped with two retractable levers located 6 cm above the floor, with a 3 x 3 cm pellet receptacle located between them. A pellet dispenser was activated by responses on the active lever, resulting in the release of a sucrose pellet into the receptacle. Each active lever response was automatically recorded using a computer interface

operating Med-IV software (Med Associates). Responses on the inactive lever were also recorded, but did not result in activation of the pellet dispenser. Each chamber was equipped with a white stimulus light, located directly above the active lever, and a white houselight.

2.5.3. Locomotor Testing Apparatus

Locomotor testing was carried out in opaque Plexiglas chambers (40 x 25 x 20 cm) with wire mesh lids. An infrared camera positioned above the chambers recorded distanced traveled (cm) using EthoVision software (Version 3, Noldus, The Netherlands).

2.5.4. Elevated Plus Maze

The EPM was equipped with two open and two closed arms (each 50 x 10 cm), radiating at 90° angles from a center platform (10 x 10 cm). The maze was elevated 115 cm above the floor and placed in a darkened room, with dim lighting provided below each of the two closed arms. A video camera was positioned to record the movement of the rat in the maze.

2.6. Procedures

2.6.1. Reinstatement of Drug Seeking

Reinstatement experiments (Exp. 1, 2, and 4) were carried out in four phases: (*1*) self-administration training [8-10 days]; (*2*) drug-free period [9-16 days]; (*3*) extinction training [3 days]; and (*4*) testing for reinstatement [2-3 days].

2.6.1.1. Phase 1: Self-Administration Training

Rats were first habituated to the self-administration chambers for a 120-min session, during which the active lever was retracted, and the inactive lever was extended. Twenty-four hours later, rats were trained to self-administer cocaine (0.23 mg/65 μl infusion, i.v.) on a FR-1 schedule of reinforcement during daily 180-min morning (2–3 h after lights off) or afternoon (6–7 h after lights off) sessions. Self-administration training alternated between morning and

afternoon sessions. An alternating procedure was implemented to ensure self-administration experience in both the morning and afternoon because extinction training spanned the entire day, and tests for reinstatement occurred exclusively in the afternoon. Rats were trained to self-administer cocaine for 8-10 days.

Each training session began with a 5-min acclimatization period, during which time the active lever was retracted, and the inactive lever was extended. The availability of cocaine was subsequently signaled by the introduction of the active lever into the chamber, illumination of a houselight (which remained lit throughout the session), and illumination of a white stimulus light above the active lever for 20 s. During the self-administration session, responses on the active lever resulted in a 3-s infusion of cocaine (in a volume of 65 μl) and 20-s illumination of the stimulus light, which represented a "time-out" period in which additional responses were recorded but not reinforced. Responses on the inactive lever were recorded throughout the 180-min session, but were without consequence.

2.6.1.2. Phase 2: Drug-Free Period

Following self-administration training, rats were kept in their home cages for a 9- (Exp. 1, 4) or 16-day (Exp. 2; extended to accommodate repeated TCAP-1 exposure) drug-free period. Rats were left undisturbed in their home cages during this time, with the exception of routine cleaning, feeding, and monitoring of weight and health. This phase was implemented to ensure that extinction and testing for reinstatement occurred beyond the acute cocaine withdrawal period, given that these studies aimed to examine the long-term reinstatement of cocaine seeking. Although this phase was considerably longer for some experiments than others, the differences in time are well-known to produce reinstatement effects of comparable magnitude (e.g., Erb et al. 2000; Erb et al. 2001).

2.6.1.3. Phase 3: Extinction Training

Following the drug-free period, rats were given three consecutive days of extinction training. Days 1 and 2 consisted of four 60-min extinction sessions, during which all conditions present during self-administration training were maintained, except that active lever responses were not reinforced by cocaine. Each 60-min session was initiated by the same events that occurred at the start of self-administration sessions. Extinction sessions were separated by 30-min intervals, during which the active lever was retracted. On Day 3 of extinction, conditions were the same as on Days 1 and 2, with two exceptions: (1) rats were given two sham injections (one i.c.v. and one i.p.), separated by 30 min, between the third and fourth extinction sessions, and (2) the fourth session began 10 min after the i.p. injection. Sham injections were given to familiarize rats with the manipulations that occurred in subsequent tests for reinstatement. Rats were subsequently assigned to pretreatment (TCAP-1 in Exp. 1; AM251 in Exp. 4) conditions, such that each group was equated according to response rates during cocaine self-administration and extinction.

2.6.1.4. Phase 4: Reinstatement Testing

Following extinction training, rats were given tests for reinstatement of cocaine seeking. At the start of each test day, rats were given three 60-min extinction sessions. Rats that exhibited 20 or fewer responses on the active lever during the second and third sessions (combined) were subsequently tested for reinstatement. Rats that did not reach this criterion were given an additional extinction session and tested the following day. Following the third extinction session, rats in Experiments 1 and 4 were pretreated (i.c.v.) with either TCAP-1 or AM251. All rats were subsequently exposed to one of the following reinstatement triggers: an injection of CRF (0.5 μg; i.c.v.), exposure to footshock stress (20 min, 0.9 mA), or an injection of cocaine (10 or 15 mg/kg, i.p.). After an additional 30 or 10 min in the CRF and cocaine conditions, respectively, or immediately after the 20 min of intermittent footshock, rats were

presented with the active lever, and non-reinforced lever responses were recorded during a 60-min test for reinstatement. In Experiments 1 and 2, rats were tested for reinstatement in response to one to three of the above triggers on up to three consecutive days, in a counterbalanced order. In Experiment 4, each rat was tested in response to a vehicle injection (i.c.v. or i.p.) and two of the three triggers on three days, in a counterbalanced order.

2.6.2. Self-Administration of Sucrose Pellets

In Experiment 5, rats were trained to self-administer sucrose pellets in order to test for non-specific motoric effects of the CB_1 receptor antagonist, AM251, on lever pressing behaviour. Rats were habituated to the sucrose self-administration chambers for a 120-min session, during which the active lever was retracted and the inactive lever extended. Twenty-four hours later, rats were trained in daily 180-min sessions to lever press for sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ, USA) on a FR-1 schedule of reinforcement for 8-10 days.

Similar to cocaine self-administration sessions, each sucrose self-administration session began with a 5-min acclimatization period, during which the active lever was retracted and the inactive lever was extended. Subsequently, the availability of sucrose pellets was signaled by the introduction of the active lever into the chamber, illumination of a houselight (which remained lit throughout the session), and illumination of a white stimulus light above the active lever for 20 s. Responses on the active lever resulted in the release of a single sucrose pellet from the pellet dispenser and 20-s illumination of the stimulus light, which represented a "time-out" period in which additional responses were recorded but not reinforced. Responses on the inactive lever were recorded throughout the 180-min session, but were without consequence. Upon reaching a stable rate of sucrose reinforcement (<10% variability in two consecutive sessions), and following a minimum of seven training sessions, rats were given an i.c.v.

injection of saline 30 min prior to the next training session to acclimatize them to manipulations that occurred in the subsequent test phase.

Tests for non-specific motoric effects of AM251 were conducted on three of the five sessions following self-administration training. Thirty min prior to each 180-min test session, every rat was injected with each dose of AM251 (0, 100, and 200 μg; i.c.v.) on separate test days in a counterbalanced order. Test sessions were separated by 48 h, with intervening baseline sucrose pellet self-administration sessions.

2.6.3. Cocaine Sensitization

Cocaine sensitization experiments (Exp. 3 and 6) were carried out in four phases: (1) habituation, (2) cocaine pre-exposure, (3) drug-free period, and (4) testing for sensitization.

2.6.3.1. Phase 1: Habituation

Rats were transported to the locomotor testing room, placed in the activity chambers for 60 min, administered an injection of saline (i.p.), and returned to the chambers for an additional 120 min. Activity was monitored both before and after the saline injection. Rats were assigned to cocaine or saline pre-exposure conditions such that each group was equated according to the average level of activity seen in the post-saline injection period.

2.6.3.2. Phase 2: Cocaine Pre-Exposure

Starting 48 h after habituation, rats were given once daily injections of cocaine or saline for 7 days. The first and last injections (0 or 15 mg/kg, i.p.) were administered in the activity chambers, immediately after a 60-min habituation period. The five intervening injections were given in the home cages (0 or 30 mg/kg, i.p.). Rats were subsequently assigned to pre-exposure (TCAP-1; Exp. 3) or pretreatment (AM251; Exp. 6A and 6B) conditions such that each group was equated according to the average level of post-injection activity on Day 7, and the ratio of this activity on Days 1 and 7 (to account for any locomotor habituation/sensitization).

2.6.3.3. Phase 3: Drug-Free Period

To parallel the drug-free periods used in the self-administration experiments (Exp. 1, 2 and 4), rats were given a drug-free period of 12 (Exp. 6A, 6B) or 19 days (Exp. 3) between cocaine exposure and testing. The drug-free period used in Experiment 3 was extended to accommodate repeated TCAP-1 exposure. Two days prior to locomotor testing, rats in Experiments 6A and 6B were given a sham i.c.v. injection to habituate them to the injection procedures used during testing. Otherwise, rats were left undisturbed in their home cages during this period, with the exception of routine cleaning, feeding, and monitoring of weight and health.

2.6.3.4. Phase 4: Testing for Sensitization

Rats in Experiment 3 were given two tests for sensitization, one in response to CRF (0.5 μg; i.c.v.) and one in response to cocaine (15 mg/kg). Rats in Experiments 6A and 6B were pretreated with AM251 (0, 10, or 100 μg; i.c.v.) 30 min prior to each of three tests for sensitization, in response to CRF, cocaine, and saline (to assess the locomotor effects of the AM251 pretreatment). Tests were separated by 48 h and given in a counterbalanced order. For each test, rats were initially placed in the activity chambers for 60 min. Following administration of all pretreatments and test substances, rats were returned to the chambers for an additional 120 min.

2.6.4. Anxiety Testing

On each of the three days prior to testing on the EPM, rats used in the anxiety studies (Exp. 7 and 8) were transported in their home cages to a darkened room adjacent the testing room, and given sham i.c.v. injections to habituate them to the procedures used during testing. On the test day, rats in all anxiety experiments were injected with AM251 (0, 10, 100, or 200 μg, i.c.v.). Thirty min later, rats in Experiments 7 were injected with CRF (0 or 0.5 μg; i.c.v.).

Rats in Experiments 8, chronically pre-exposed to either cocaine or saline, were injected with saline (i.p.) 30 min following the AM251 pretreatment to potentiate the withdrawal state in those pre-exposed to cocaine. After an additional 30 min, all rats were placed individually onto the center platform of the EPM, facing towards a closed arm, and allowed to explore the maze freely for 5 min. Video footage of the testing was captured, and the entries made into, and time spent in, the open and closed arms was later scored by an observer blind to the treatments. Rats were considered within an arm when both front paws and at least one rear paw were within the boundaries of the arm. Following each test, the maze was cleaned using a 70% ethanol solution, and allowed to dry.

2.6.5. Corticosterone Radioimmunoassay

Blood plasma from rats in Experiments 7B and 8B was analyzed for corticosterone content using a double-antibody ^{125}I Radioimmunoassay (ImmuChem 07-120103, MP Biomedicals, Orangeburg, NY). Using a plasma pool from representative rats in these experiments, parallelism was confirmed between the standard curve and serially diluted plasma. Manufacturer's directions were followed except that the volumes of all reagents were halved and plasma diluted 1:300 (0.16 μL of plasma per tube). Each sample was measured in duplicate.

2.7. Data Analysis

2.7.1. Reinstatement of Drug Seeking

In Experiments 1 and 2, the main dependent measures were the number of responses on the active and inactive levers during the extinction session preceding (EXT) and reinstatement test following the test challenges (CRF, Footshock, Cocaine). Separate analyses were carried out for each test condition, using repeated measures ANOVAs for the between-subjects factor of

TCAP-1 (0, 300 pmol [Exp. 1]; 0-600 pmol [Exp. 2]), and the within-subjects factor of Test (EXT, CRF/Footshock/Cocaine). Significant interactions were followed by LSD post-hoc comparisons ($p<.05$), as appropriate.

In Experiment 2, the number of responses on the active lever during each extinction day was also analyzed using a repeated measures ANOVA for the between-subjects factor of TCAP-1 [0-600 pmol] and the within-subjects factor of Day (1-3).

In Experiment 4, the main dependent measures were the number of responses on the active and inactive levers in response to the test challenges (CRF, Footshock, and Cocaine) and their vehicle conditions (VEH). Separate analyses were carried out for each test condition, using repeated measures ANOVAs for the between-subjects factor of AM251 (0, 10, 100, 200 μg), and the within-subjects factor of Test (VEH, CRF/Footshock/Cocaine). Significant interactions were followed by LSD post-hoc comparisons ($p<.05$), as appropriate.

2.7.2. Self-Administration of Sucrose Pellets

In Experiment 5, the main dependent measures were the number of responses (reinforced and total) on the active and inactive levers in response to AM251 treatment. A repeated measures ANOVA was conducted to compare the effects of each dose of AM251 (0, 100, 200 μg) on sucrose pellet self-administration.

2.7.3. Cocaine Sensitization

In Experiments 3 and 6, the main dependent measures were the distanced traveled (cm) during the 60 min prior to, and 120 min following, the CRF or cocaine challenges. In Experiment 6, an additional dependent measure of the distance travelled prior to, and following, a saline challenge was used. Separate analyses were carried out for each challenge, using two-way ANOVA for the factors of Cocaine Pre-Exposure (Cocaine, Saline), TCAP-1 Pre-Exposure (0, 300 pmol [Exp. 3]), and AM251 (0, 10, 100 μg [Exp. 6]). Significant interactions were followed by LSD post-hoc comparisons ($p<.05$), as appropriate.

2.7.4. Anxiety Testing and Corticosterone Radioimmunoassay

In Experiments 7A and 8A, the dependent measures for anxiety testing were time spent in the open arms, number of open arm entries, number of closed arm entries, total number of arm entries, and percent ratio of open to closed arm entries in the EPM. In Experiments 7B and 8B, the dependent measure for the corticosterone radioimmunoassay was the concentration of trunk plasma corticosterone (ng/ml). Separate analyses were carried out for each measure, using two-way ANOVAs for the factors of AM251 (0, 10, 100, 200 μg) and CRF (0, 0.5 μg) in Experiments 7A and 7B, and AM251 (0, 10, 100 μg) and Pre-Exposure (Cocaine, Saline) in Experiments 8A and 8B. Significant effects were followed by LSD post-hoc comparisons ($p<.05$), as appropriate. Pearson correlation coefficients ($p<.05$) were also calculated between each EPM measure and plasma corticosterone levels.

CHAPTER 3

TCAP-1 Regulation of the Reinstatement of Cocaine Seeking and Cocaine Sensitization

CHAPTER 3

TCAP-1 Regulation of the Reinstatement of Cocaine Seeking and Cocaine Sensitization

3.1. Introduction

In recent years, the bioactive peptide, TCAP-1, has emerged as an important regulator of stress and anxiety (Qian et al., 2004; Wang et al., 2005b). As described in Chapter 1, TCAP-1 shares about 20% of its primary sequence identity with CRF (Lovejoy et al., 2006), and is widely expressed within forebrain and limbic regions rich with CRF neurons and receptors (Aguilera et al., 2004), such as the hippocampus, CeA, BLA, ventromedial nucleus of the hypothalamus, piriform cortex, and BNST (Wang et al., 2005b). Although the cellular action of TCAP-1 appears to be independent of CRF receptor transmission (Wang et al., 2005b; Al Chawaf et al., 2007b; De Almeida et al., 2011), TCAP-1 treatment modulates many intracellular systems that are similarly recruited by CRF (e.g. cAMP, CREB, ERK; Grammatopoulos and Chrousos, 2002; Stern et al., 2011; Chand et al., 2011). Collectively, these findings suggest that TCAP-1 may be uniquely positioned to regulate the effects of stress via an interaction with the CRF system.

Also as discussed in Chapter 1, synthetic TCAP-1 modulates several behavioural and neuronal effects of CRF in a primarily, though not exclusively (Tan et al., 2008), inhibitory manner. Briefly, 5 days of i.v. TCAP-1 injections decreased CRF-induced anxiety in the EPM and open field (Al Chawaf et al., 2007b), and 5 days of i.c.v. TCAP-1 injections blocked CRF-induced increases in acoustic startle responses (Tan et al., 2008). Furthermore, acute treatment with i.c.v. TCAP-1 attenuated CRF-induced expression of c-fos, a widely used marker of neuronal activation (Curran and Morgan, 1995), in the hippocampus, amygdala, and other limbic brain regions (Tan et al., 2009). Based on these and similar findings, it has been proposed that TCAP-1 may act downstream of CRF receptor activation to modify neuronal function in a manner that serves to inhibit certain cellular and behavioural effects of CRF.

Given the important role of CRF in mediating some long-lasting behavioural effects of cocaine (see Chapter 1), the known neuroanatomical and functional convergence of central TCAP-1 and CRF systems, and the evidence that TCAP-1 treatment can alter behavioural responses to CRF, it is plausible that TCAP-1 could act to modulate the expression of cocaine-related behaviours. Thus, the experiments presented in this chapter (Experiments 1-3) were designed to explore the effects of TCAP-1 on the reinstatement of cocaine seeking and the expression of cocaine-induced behavioural sensitization. First, the effects of acute TCAP-1 pretreatment (Experiment 1) and repeated (5-day) TCAP-1 pre-exposure (Experiment 2) on the reinstatement of cocaine seeking induced by i.c.v. injections of CRF, exposure to intermittent footshock stress, and priming injections of cocaine, were assessed. Both acute and repeated treatments with TCAP-1 have been shown to modify stress-related behaviour, and so it was important to assess the effects of both regimens on reinstatement behaviour. In general, however, the behavioural effects of TCAP-1 are more uniform and pronounced with repeated treatment, especially those on the behavioural responses to CRF. Accordingly, in the present experiments, repeated, but not acute, TCAP-1 treatment selectively interfered with CRF-induced reinstatement of cocaine seeking. On the basis of these findings, Experiment 3 was subsequently carried out to test the effects of repeated TCAP-1 pre-exposure on the expression of cocaine-induced behavioural sensitization to challenge injections of CRF and cocaine. In a manner paralleling the findings from Experiment 2, repeated exposure to TCAP-1 selectively blocked the effects of CRF on the expression of cocaine-induced behavioural sensitization.

3.2. Experiment 1: Effects of Acute TCAP-1 on the Reinstatement of Cocaine Seeking

3.2.1. Materials and Methods

3.2.1.1. Subjects

A total of 23 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

3.2.1.2. Surgery

Rats were surgically implanted with both an i.v. catheter and i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2).

3.2.1.3. Apparatus

All behavioural procedures were carried out in the drug self-administration chambers (Med Associates) described in Chapter 2 (Section 2.5.1).

3.2.1.4. Drugs

Cocaine HCl (Medisca Pharmaceuticals), TCAP-1 (American Peptide Company) and CRF (Sigma-Aldrich) were dissolved in physiological saline, which served as the vehicle in this experiment. The TCAP-1 dose of 300 pmol was selected based on previous reports showing attenuation of CRF-induced expression of c-fos in limbic brain regions, and modulation anxiety-related behaviour in the EPM by comparable TCAP-1 doses (Tan et al., 2009). Central injections of TCAP-1 and CRF were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

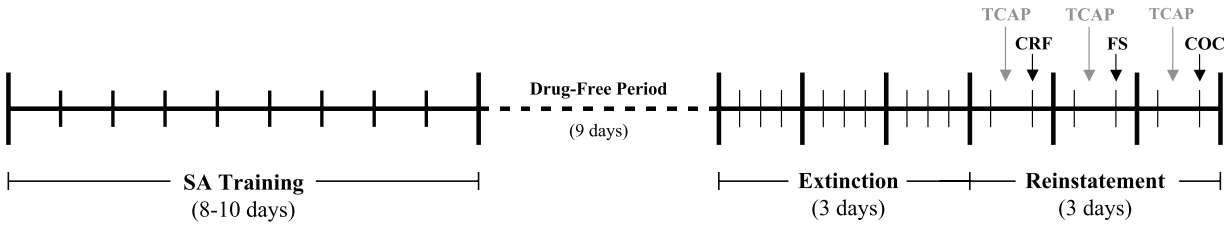
3.2.1.5. Reinstatement Procedures

Figure 6 shows the procedural timeline of Experiment 1.

3.2.1.5.1. Phase 1: Self-Administration

Rats were trained to self-administer cocaine (0.23 mg/65 µl infusion, i.v.) for a total of 8-10 days using the procedures described in Chapter 2 (Section 2.6.1.1).

Figure 6



Procedural timeline for Experiment 1.

3.2.1.5.2. Phase 2: Drug-Free Period

Rats were maintained in their home cages for 9 days under the vivarium conditions described in Chapter 2 (Section 2.6.1.2).

3.2.1.5.3. Phase 3: Extinction

Extinction Days 1-3 were carried out as described in Chapter 2 (Section 2.6.1.3).

3.2.1.5.4. Phase 4: Tests for Reinstatement

Following extinction, rats were tested for CRF-, footshock- and cocaine-induced reinstatement, as described in Chapter 2 (Section 2.6.1.4). At the start of each test day, rats were given three 60-min extinction sessions. Subsequently, rats were given an injection of TCAP-1 (0, or 300 pmol; i.c.v.), 30 min prior to an injection of CRF (0.5 μg; i.c.v.), exposure to intermittent footshock stress (20 min; 0.9 mA), or an injection of cocaine (15 mg/kg; i.p.). Thirty, 0, or 10 min following the CRF, footshock, or cocaine, respectively, tests for reinstatement began, whereby the previously drug-reinforced lever was extended into the chamber and responding was recorded over a 60-min period. Different groups of rats were pretreated with the 0 and 300 pmol dose of TCAP-1, but each rat was tested after each reinstatement trigger on consecutive days, in a counterbalanced order. Importantly, early pilot work revealed that acute administration of 300 pmol TCAP-1 did not induce reinstatement of cocaine seeking. As such, the present experiment did not include a vehicle test condition, but instead used responding on the active lever following exposure to CRF, footshock, or cocaine, relative to that seen in the preceding extinction session, as a measure of the reinstatement of cocaine seeking.

3.2.2. Results

3.2.2.1. Self-Administration

Once rats acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period. The mean ± standard error of the mean (SEM) number of infusions made during the 180-min sessions on the last two days of training was 35.20 ± 2.34 and 37.00 ± 2.70, respectively, corresponding to average daily cocaine intake of 8.10 ± 0.53 and 8.51 ± 0.62 mg.

3.2.2.2. Extinction

At the start of the extinction phase, rats showed characteristic heightened responding on the previously drug-reinforced lever, averaging 88.52 ± 11.05 in the first 60-min extinction session. This rate of responding gradually declined to 5.48 ± 1.28 active lever responses in the final 60-min session on Day 3 of extinction.

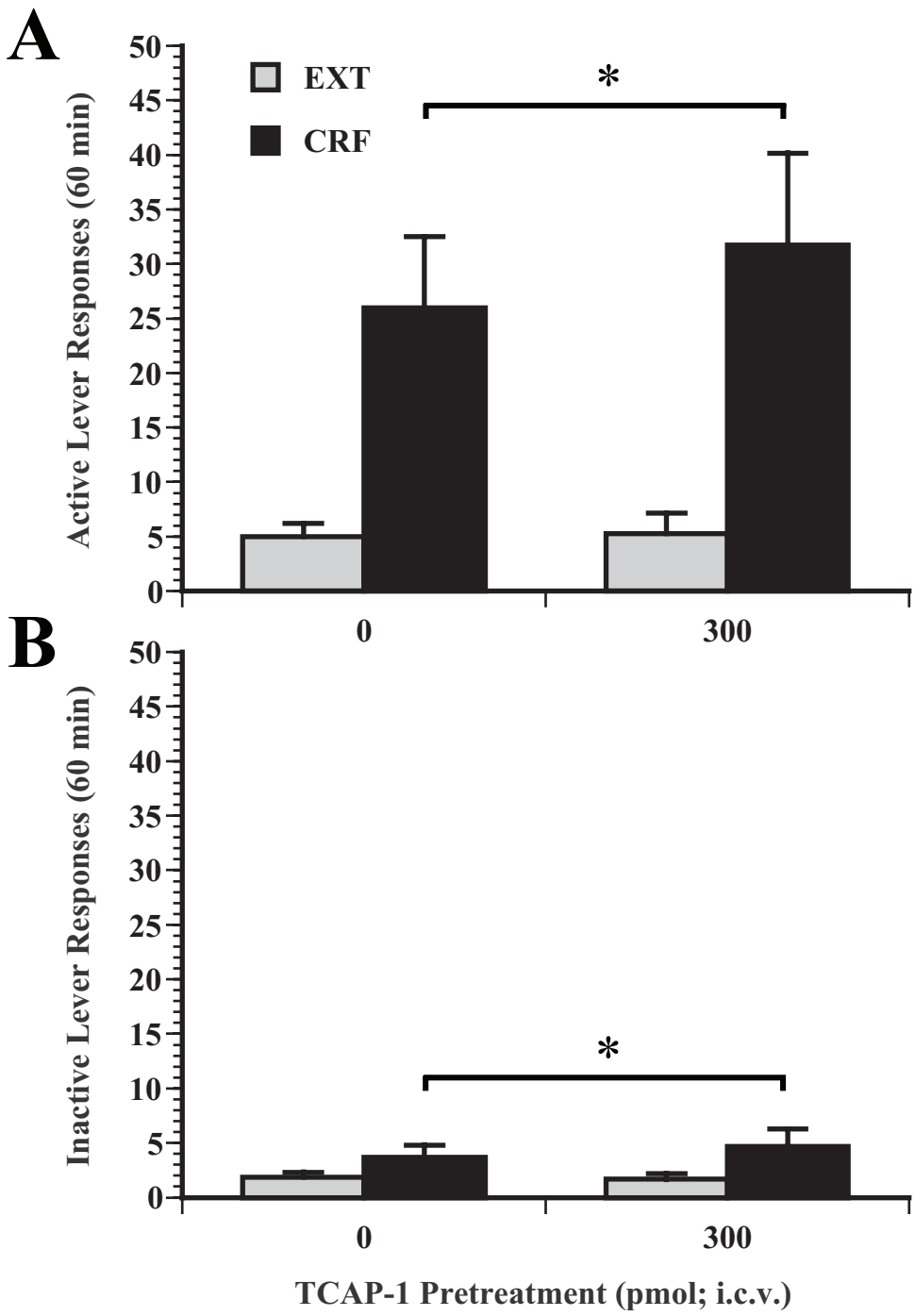
3.2.2.3. Tests for Reinstatement

Figure 7 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test session following TCAP-1 pretreatment (0 or 300 pmol; i.c.v.) and CRF treatment (0.5 μg; i.c.v.). A repeated measures ANOVA of active lever responding revealed only a significant main effect of CRF [$F(1,20)=21.14$, $p<.001$]. Indeed, CRF induced a higher level of responding on the active lever, relative to that seen during the preceding extinction session, irrespective of TCAP-1 pretreatment. Thus, acute TCAP-1 pretreatment had no effect on CRF-induced reinstatement. A repeated measures ANOVA of inactive lever responding revealed a similar main effect of CRF [$F(1,20)=6.22$, $p<.05$], reflecting slightly higher levels of responding following CRF relative to that seen in the preceding extinction session. However, responding on the inactive lever was very low (less than 20% of active lever responding) across all pretreatment and test conditions.

Figure 8 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test session following, TCAP-1 pretreatment (0 or 300 pmol; i.c.v.) and footshock stress (20 min; 0.9 mA). A repeated measures ANOVA of active lever responding revealed only a significant main effect of Footshock [$F(1,19)=26.85$, $p<.001$]. Similar to CRF, footshock induced a higher level of responding on the active lever, relative to that seen during the preceding extinction session, irrespective of TCAP-1 pretreatment. Thus, acute TCAP-1 pretreatment also had no effect on footshock-induced reinstatement. A repeated measures ANOVA of inactive lever responding revealed a similar main effect of Footshock [$F(1,19)=6.70$, $p<.05$], reflecting slightly higher levels of responding following footshock relative to that seen in the preceding extinction session. However, responding on the inactive lever was very low (less than 15% of active lever responding) across all pretreatment and test conditions.

Figure 9 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test session following, TCAP-1 (0 or 300 pmol; i.c.v.) pretreatment and cocaine treatment (15 mg/kg; i.p.). A repeated measures ANOVA revealed only a significant main effect of Cocaine [$F(1,19)=56.91$, $p<.001$]. Similar to CRF and footshock, cocaine induced a higher level of responding on the active lever, relative to that seen during the preceding extinction session, irrespective of TCAP-1 pretreatment. Thus, acute TCAP-1 pretreatment also had no effect on cocaine-induced reinstatement. A repeated measures ANOVA of inactive lever responding revealed a similar main effect of Cocaine [$F(1,19)=8.91$, $p<.05$], reflecting slightly higher levels of responding following cocaine relative to that seen in the preceding extinction session. However, responding on the inactive lever was very low (less than 15% of active lever responding) across all pretreatment and test conditions.

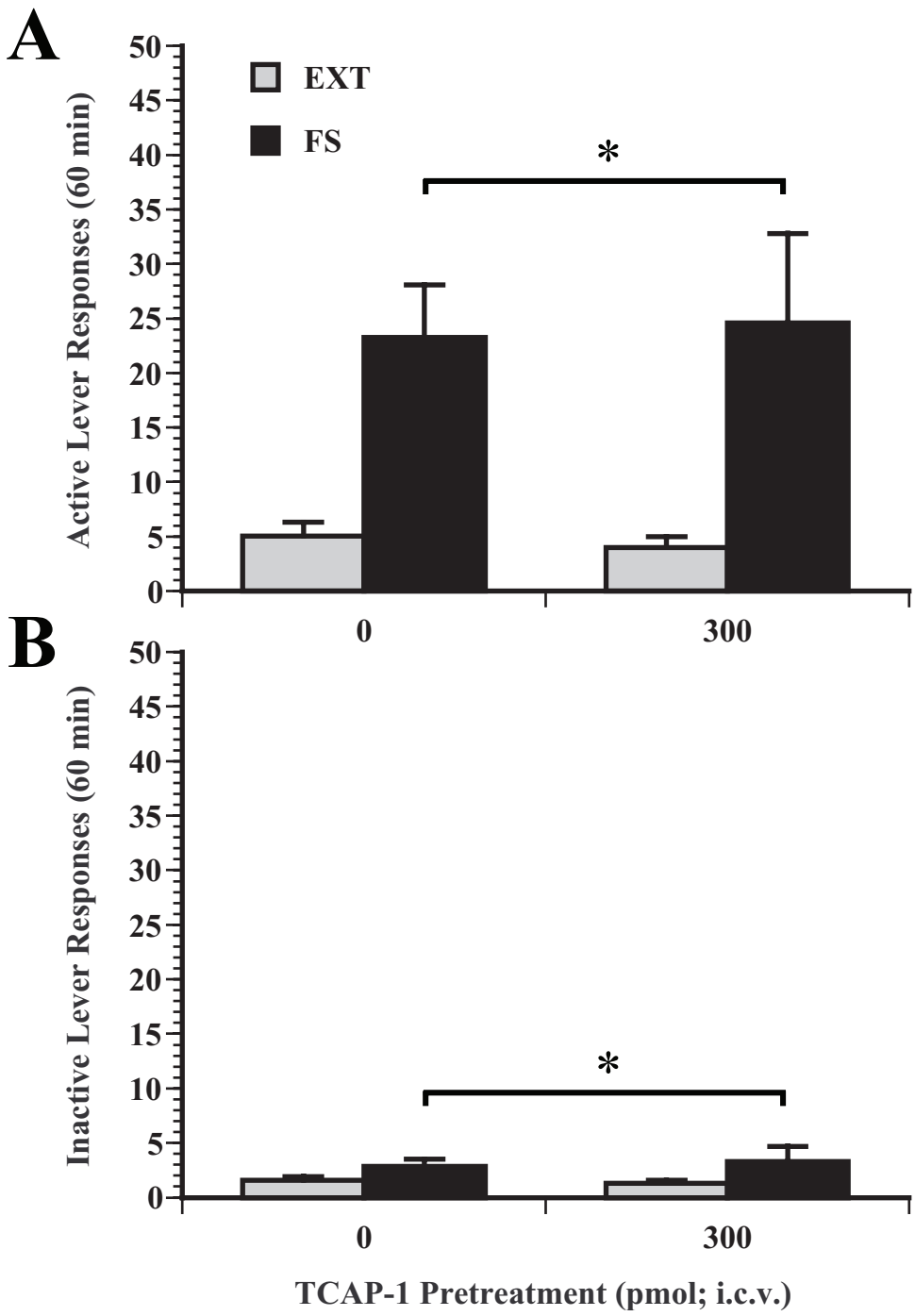
Figure 7



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test session following TCAP-1 pretreatment (0 or 300 pmol; i.c.v.) and CRF treatment (0.5 µg; i.c.v.) [Experiment 1].
(A) Repeated measures ANOVA: Main effect of CRF [$F(1,20)=21.14$, $p<.001$]. (B) Repeated measures ANOVA: Main effect of CRF [$F(1,20)=6.22$, $p<.05$].

* Different from EXT condition, $p<.05$.

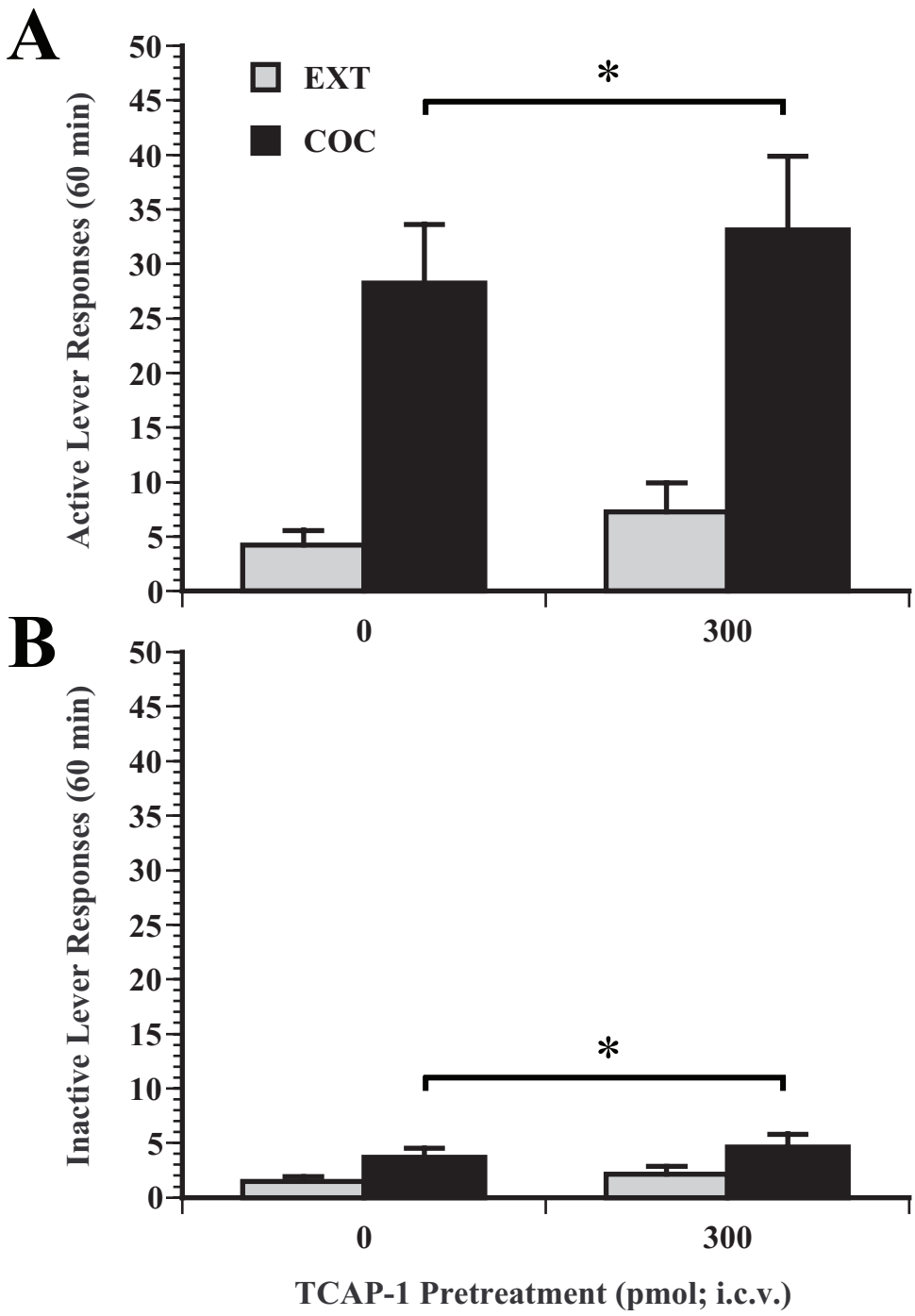
Figure 8



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test session following, TCAP-1 pretreatment (0 or 300 pmol; i.c.v.) and footshock stress (FS; 20 min; 0.9 mA) [Experiment 1]. (A) Repeated measures ANOVA: Main effect of FS [$F(1,19)=26.85$, $p<.001$]. (B) Repeated measures ANOVA: Main effect of FS [$F(1,19)=6.70$, $p<.05$].

* Different from EXT condition, $p<.05$.

Figure 9



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test session following, TCAP-1 (0 or 300 pmol; i.c.v.) pretreatment and cocaine treatment (COC;15 mg/kg; i.p.) [Experiment 1]. (A) Repeated measures ANOVA: Main effect of COC [$F(1,19)=56.91$, $p<.001$]. (B) Repeated measures ANOVA: Main effect of COC [$F(1,19)=8.91$, $p<.05$].

* Different from EXT condition, $p<.05$.

3.3. Experiment 2: Effects of Repeated TCAP-1 on the Reinstatement of Cocaine Seeking

3.3.1. Materials and Methods

3.3.1.1. Subjects

A total of 42 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

3.3.1.2. Surgery

Rats were surgically implanted with both an i.v. catheter and i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2).

3.3.1.3. Apparatus

All behavioural procedures were carried out in the drug self-administration chambers (Med Associates) described in Chapter 2 (Section 2.5.1).

3.3.1.4. Drugs

Cocaine HCl (Medisca Pharmaceuticals), TCAP-1 (American Peptide Company) and CRF (Sigma-Aldrich) were dissolved in physiological saline, which served as the vehicle in this experiment. The TCAP-1 doses of 30, 300 and 600 pmol were selected based on previous reports showing that repeated (5-day) exposure to comparable doses of TCAP-1 interferes with CRF-induced anxiety in the open field and EPM (Al Chawaf et al., 2007b), and CRF-induced increases in acoustic startle responses (Tan et al., 2008). Central injections of TCAP-1 and CRF were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

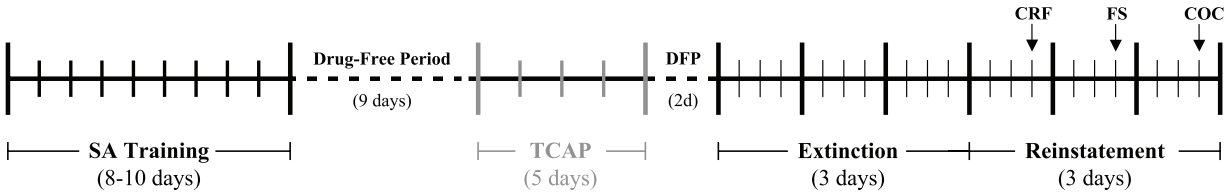
3.3.1.5. Reinstatement Procedures

Figure 10 shows the procedural timeline of Experiment 2.

3.3.1.5.1. Phase 1: Self-Administration

Rats were trained to self-administer cocaine (0.23 mg/65 µl infusion, i.v.) for a total of 8-10 days using the procedures described in Chapter 2 (Section 2.6.1.1).

Figure 10



Procedural timeline for Experiment 2.

3.3.1.5.2. Phase 2: Drug-Free Period

Rats were maintained in their home cages for 16 days under the vivarium conditions described in Chapter 2 (Section 2.6.1.2).

3.3.1.5.3. Phase 3: TCAP-1 Pre-Exposure

Beginning nine days into the 16-day drug-free period, rats were given five daily injections of TCAP-1 (0, 30, 300 or 600 pmol, i.c.v.) in their home cages. Initially, rats were pre-exposed to either 0 or 300 pmol TCAP-1, and tested for reinstatement under three different conditions (see Section 3.3.1.5.5). Based on the effects of these doses, higher (600 pmol) and lower (30 pmol) dose groups were added in subsequent replications to achieve meaningful dose response profiles for each test condition (*note*: additional rats in the original dose groups were included in each replication).

3.3.1.5.4. Phase 4: Extinction

Extinction Days 1-3 were carried out as described in Chapter 2 (Section 2.6.1.3).

3.3.1.5.5. Phase 5: Tests for Reinstatement

Following extinction, rats were tested for CRF-, footshock- and cocaine-induced reinstatement, as described in Chapter 2 (Section 2.6.1.4). At the start of each test day, rats were given three 60-min extinction sessions. Subsequently, rats were given an injection of CRF (0.5 μg; i.c.v.), exposed to intermittent footshock stress (20 min; 0.9 mA), or given a priming injection of cocaine (15 mg/kg; i.p.). Thirty, 0, or 10 min following the CRF, footshock or cocaine, respectively, tests for reinstatement began, whereby the previously drug-reinforced lever was extended into the chamber and responding was recorded over a 60-min period. Different groups of rats were pretreated with different doses of TCAP-1. Each rat was tested on 1-3 of the different reinstatement triggers on consecutive days, in a counterbalanced order. As in Experiment 1, responding on the active lever following exposure to CRF, footshock, or

cocaine, relative to that seen in the preceding extinction session, was used as a measure of the reinstatement of cocaine seeking.

3.3.2. Results

3.3.2.1. Self-Administration

Once rats acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period. The mean ± SEM number of infusions made by each TCAP-1 pre-exposure group during the 180-min sessions on the last two days of training, respectively, was 34.88 ± 2.21 and 36.13 ± 2.99 (0 pmol TCAP-1), 36.83 ± 2.52 and 39.17 ± 1.98 (30 pmol TCAP-1), 37.90 ± 2.97 and 39.90 ± 2.75 (300 pmol TCAP-1), and 35.00 ± 2.97 and 39.10 ± 3.41 (600 pmol TCAP-1).

3.3.2.2. Extinction

Figure 11 shows the mean (±SEM) total number of responses on the active lever during each of three consecutive days of extinction (averaged across four sessions) following pre-exposure to vehicle or TCAP-1. A repeated measures ANOVA of active lever responding revealed only a significant main effect of Day [$F(2,72)=118.89$, $p<.001$]. Thus, TCAP-1 pre-exposure did not alter the time-course of extinction responding.

3.3.2.3. Tests for Reinstatement

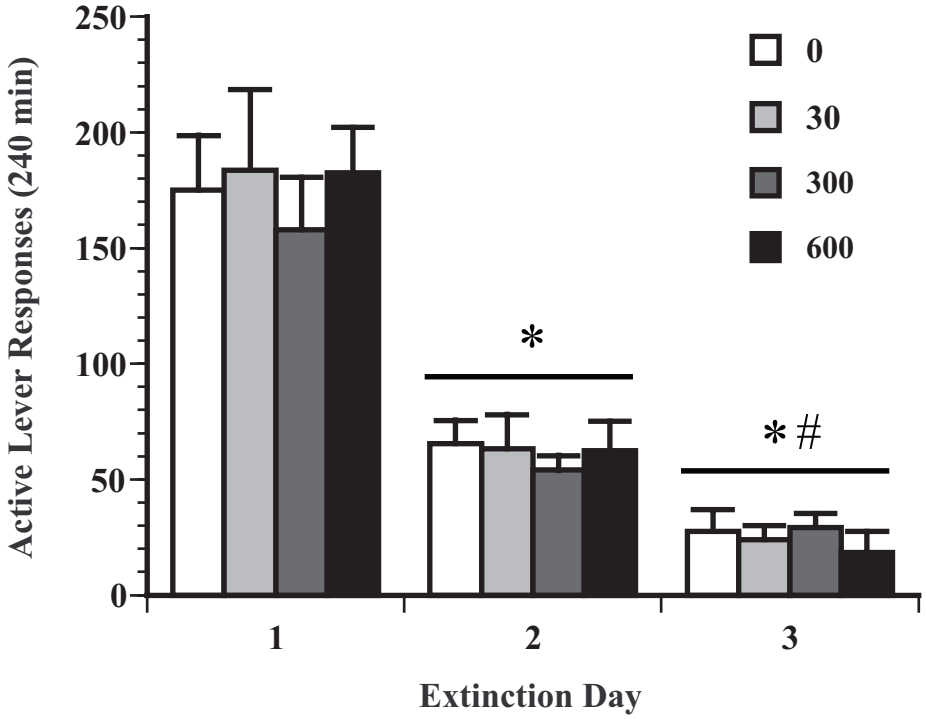
Figure 12 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test following, CRF treatment (0.5 μg; i.c.v.) by rats repeatedly pre-exposed to TCAP-1 (0, 30, or 300 pmol; i.c.v.). It can be seen that, across the range of doses tested, TCAP-1 pre-exposure blocked CRF-induced reinstatement of cocaine seeking. Indeed, a repeated measures ANOVA of total responses on the active lever responding revealed a significant main effect of CRF [$F(1,25)=5.78$, $p<.05$] and TCAP-1 x CRF interaction [$F(2,25)=5.25$, $p<.05$]. CRF induced a

higher level of responding on the active lever in rats pre-exposed to 0 pmol TCAP-1, relative to that seen during the preceding extinction session ($p<.005$), but not in rats pre-exposed to 30 or 300 pmol TCAP-1. Rats pre-exposed to 0 pmol TCAP-1 also responded significantly more on the active lever following CRF treatment than those pre-exposed to 30 or 300 pmol TCAP-1 ($p's<.01$). Finally, a repeated measures ANOVA of inactive lever responding revealed no significant effects. Indeed, responding on the inactive lever was very low (less than 25% of active lever responding) across all pre-exposure and test conditions.

Figure 13 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test session following, footshock stress (20 min; 0.9 mA) made by rats repeatedly pre-exposed to TCAP-1 (0, 300, or 600 pmol; i.c.v.). In this case, a repeated measures ANOVA of active lever responding revealed only a significant main effect of Footshock [$F(1,30)=45.25$, $p<.001$]. Indeed, irrespective of TCAP-1 pre-exposure, footshock induced a higher level of responding on the active lever, relative to that seen during the preceding extinction session. Thus, whereas repeated TCAP-1 pre-exposure blocked the effect of i.c.v. CRF on reinstatement of cocaine seeking, it was without effect on reinstatement induced by footshock stress. A repeated measures ANOVA of inactive lever responding revealed a similar main effect of Footshock [$F(1,30)=8.28$, $p<.01$], reflecting slightly higher levels of responding following footshock relative to that seen in the preceding extinction session. However, responding on the inactive lever was very low (less than 20% of active lever responding) across all pre-exposure and test conditions.

Figure 14 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction session preceding, and 60-min reinstatement test session following, cocaine treatment (15 mg/kg; i.p.) made by rats repeatedly pre-exposed to TCAP-1 (0, 300, or 600 pmol; i.c.v.). Similar to the footshock condition, a repeated measures ANOVA

Figure 11

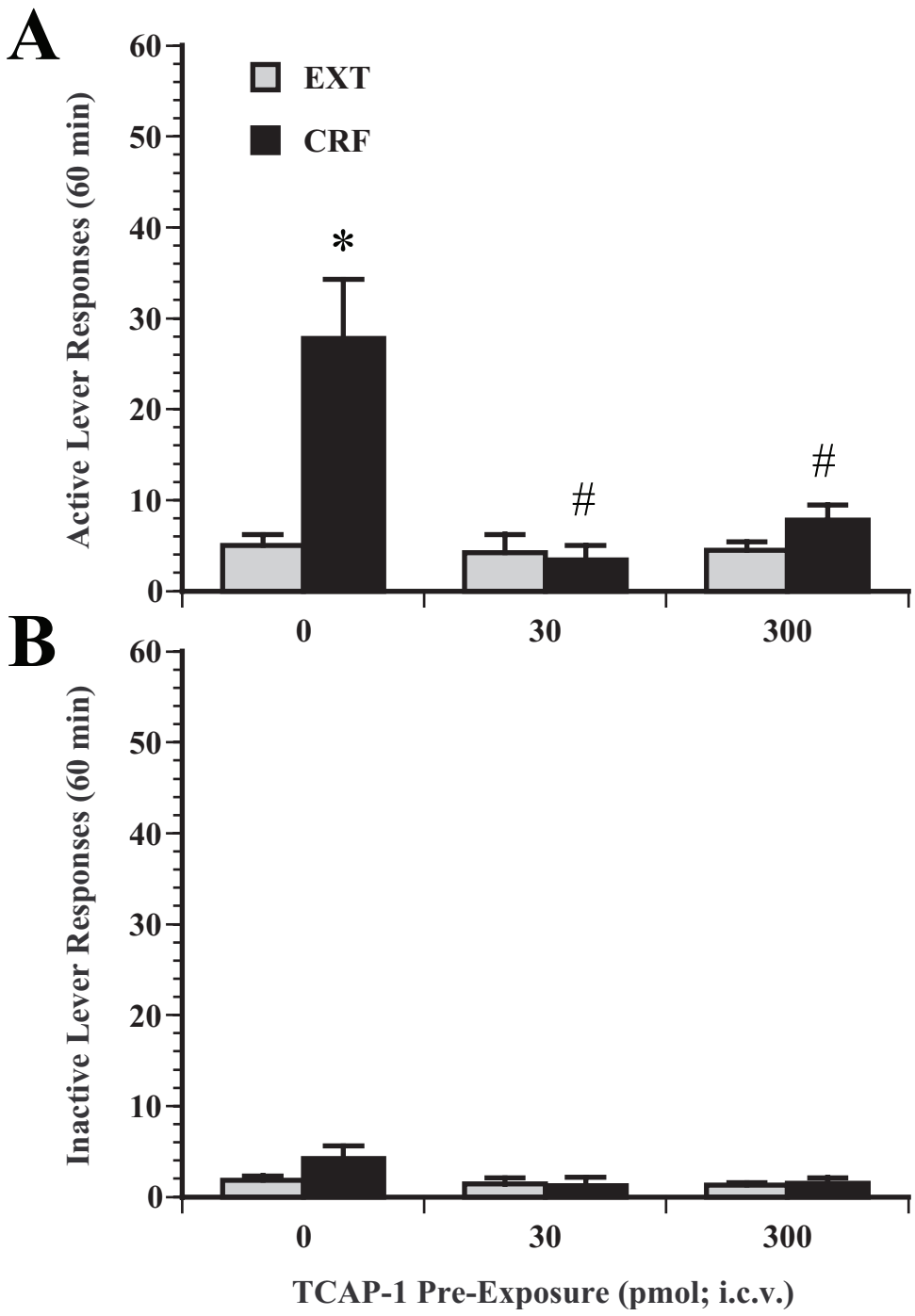


Mean (±SEM) total number of responses on the active lever during each of three consecutive days of extinction (averaged across four sessions) following repeated pre-exposure to TCAP-1 (0, 30, 300, or 600 pmol; i.c.v.) [Experiment 2].

Repeated measures ANOVA: Main effect of Day [$F(2,72)=118.89$, $p<.001$]; LSD post-hoc.

* Different from Extinction Day 1, $p<.05$; # Different from Extinction Day 2, $p<.05$.

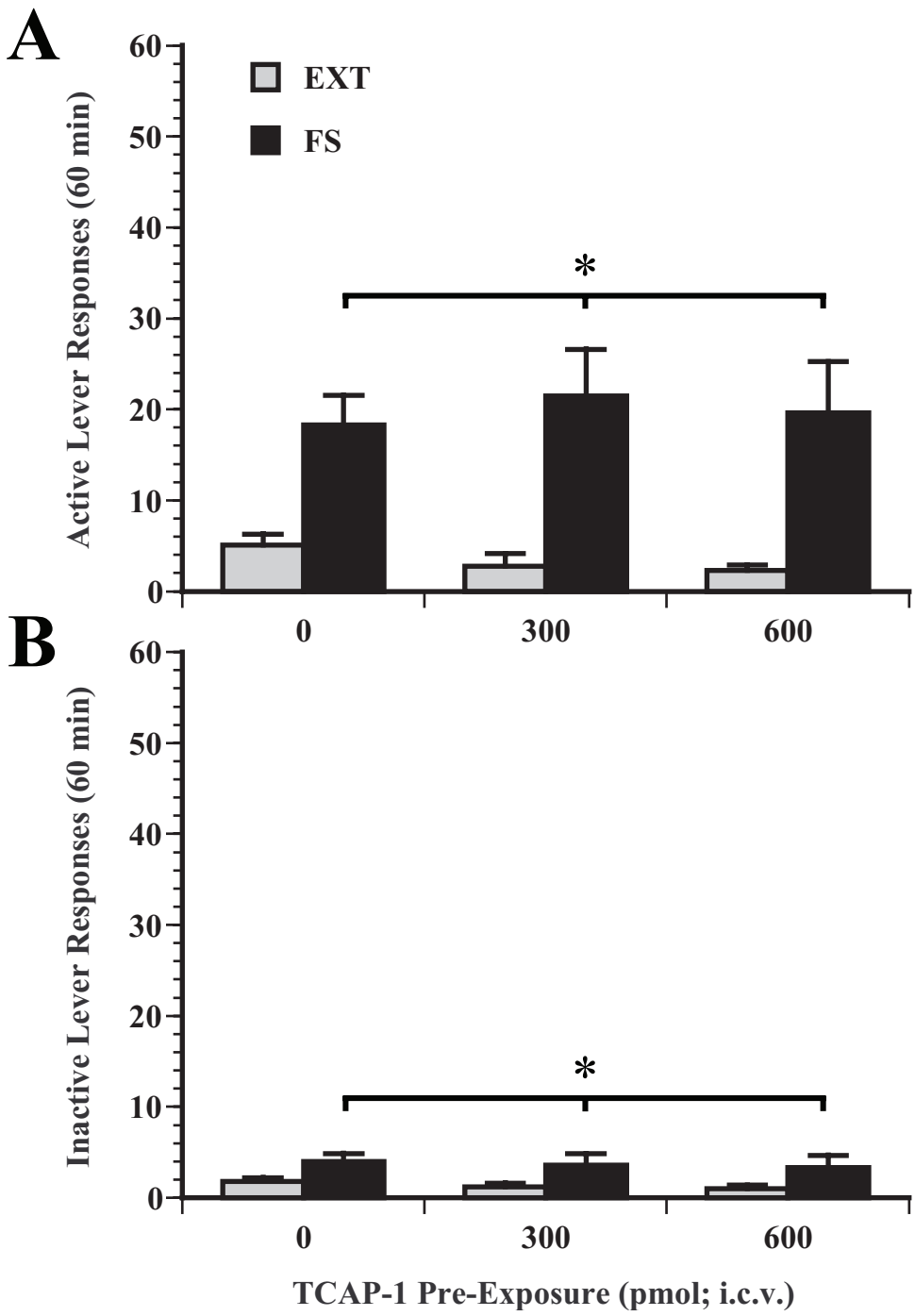
Figure 12



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test following, CRF treatment (0.5 µg; i.c.v.) by rats repeatedly pre-exposed to TCAP-1 (0, 30, or 300 pmol; i.c.v.) [Experiment 2]. (A) Repeated measures ANOVA: Main effect of CRF [$F(1,25)=5.78$, $p<.05$], TCAP-1 x CRF interaction [$F(2,25)=5.25$, $p<.05$]; LSD post-hoc.

* Different from EXT condition, $p<.05$; # Different from 0 pmol TCAP-1 condition, $p<.05$.

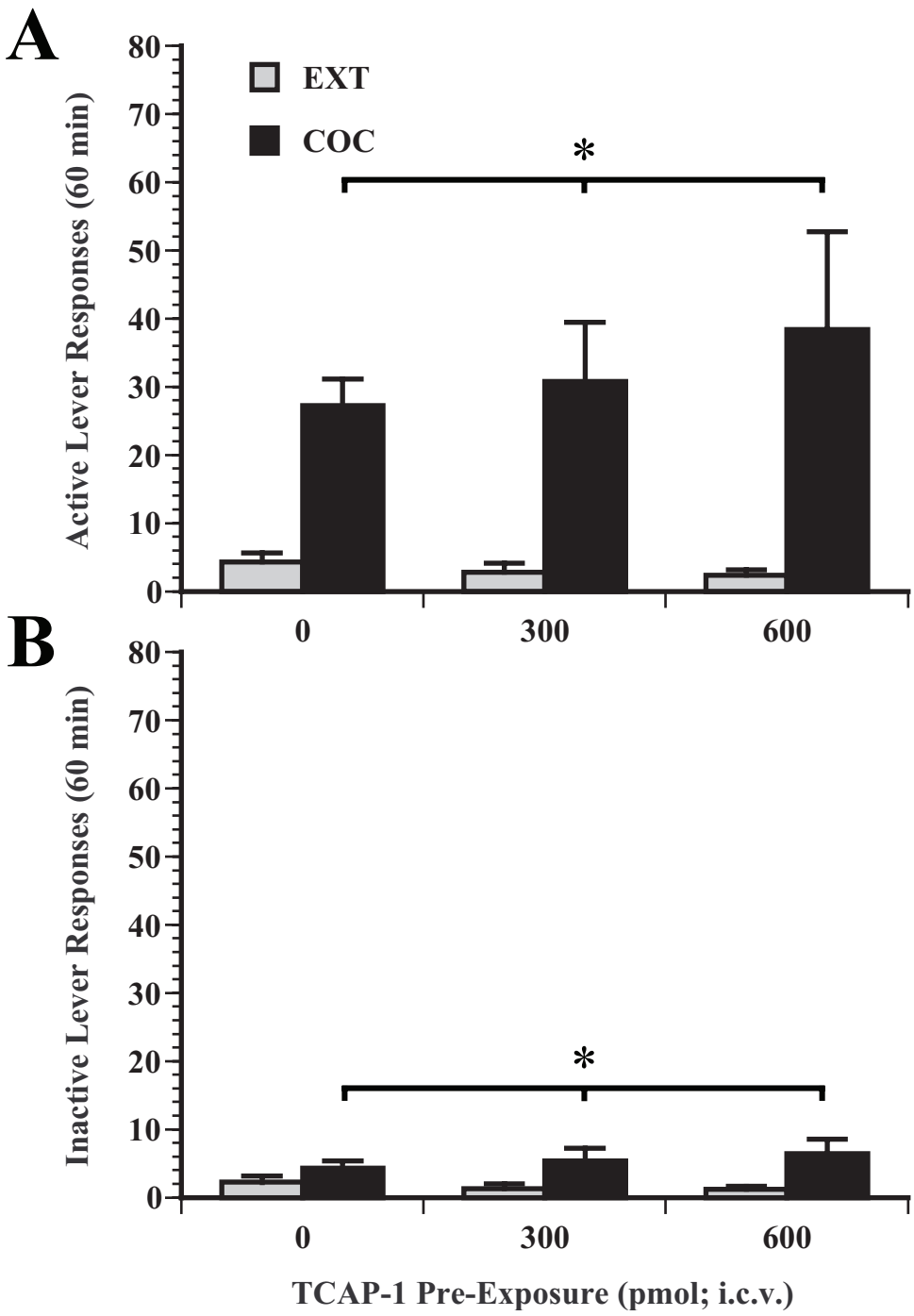
Figure 13



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test session following, footshock stress (20 min; 0.9 mA) made by rats repeatedly pre-exposed to TCAP-1 (0, 300, or 600 pmol; i.c.v.) [Experiment 2]. (A) Repeated measures ANOVA: Main effect of FS [$F(1,30)=45.25$, $p<.001$]. (B) Repeated measures ANOVA: Main effect of FS [$F(1,30)=8.28$, $p<.01$].

* Different from EXT condition, $p<.01$.

Figure 14



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during the 60-min extinction (EXT) session preceding, and 60-min reinstatement test session following, cocaine treatment (15 mg/kg; i.p.) made by rats repeatedly pre-exposed to TCAP-1 (0, 300, or 600 pmol; i.c.v.) [Experiment 2]. (A) Repeated measures ANOVA: Main effect of COC [$F(1,29)=36.39$, $p<.001$]. (B) Repeated measures ANOVA: Main effect of COC [$F(1,29)=12.50$, $p<.001$].

* Different from EXT condition, $p<.001$.

of active lever responding revealed only a main effect of Cocaine [$F(1,29)=36.39$, $p<.001$]. Again, irrespective of TCAP-1 pre-exposure, cocaine induced a higher level of responding on the active lever, relative to that seen during the preceding extinction session. Thus, repeated TCAP-1 pre-exposure had no effect on cocaine-induced reinstatement. A repeated measures ANOVA of inactive lever responding also revealed only main effect of Cocaine [$F(1,29)=12.50$, $p<.001$], reflecting slightly higher levels of responding following cocaine relative to that seen in the preceding extinction session. However, responding on the inactive lever was very low (less than 20% of active lever responding) across all pre-exposure and test conditions.

3.4. Experiment 3: Effects of Repeated TCAP-1 on Cocaine Sensitization

3.4.1. Materials and Methods

3.4.1.1. Subjects

A total of 60 male Wistar rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

3.4.1.2. Surgery

Rats were surgically implanted with an i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2.1).

3.4.1.3. Apparatus

All behavioural procedures were carried out in the locomotor testing apparatus described in Chapter 2 (Section 2.5.3).

3.4.1.4. Drugs

Cocaine HCl (Medisca Pharmaceuticals), CRF (Sigma-Aldrich), and TCAP-1 (American Peptide Company) were dissolved in physiological saline, which served as the vehicle in this experiment. The TCAP-1 dose of 300 pmol was selected based on my previous findings (Exp. 2), as well as previous reports showing that repeated exposure to comparable doses of TCAP-1

interferes with CRF-induced anxiety in the open field and EPM (Al Chawaf et al., 2007b), and CRF-induced increases in acoustic startle responses (Tan et al., 2008). Central injections of TCAP-1 and CRF were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

3.4.1.5. Sensitization Procedures

Figure 15 shows the procedural timeline of Experiment 3.

3.4.1.5.1. Phase 1: Habituation

Rats were transported to the locomotor testing room, injected with saline (i.p.), and assessed for basal activity levels, as described in the Chapter 2 (Section 2.6.3.1).

3.4.1.5.2. Phase 2: Cocaine Pre-Exposure

Starting 48 h after habituation, rats were given once daily injections of cocaine or saline for seven days. The first and last injections (0 or 15 mg/kg, i.p.) were administered in the activity chambers, immediately after a 60-min habituation period. The five intervening injections were given in the home cages (0 or 30 mg/kg, i.p.).

3.4.1.5.3. Phase 3: Drug-Free Period

Rats were maintained in their home cages for 19 days under the vivarium conditions described in Chapter 2 (Section 2.6.3.3).

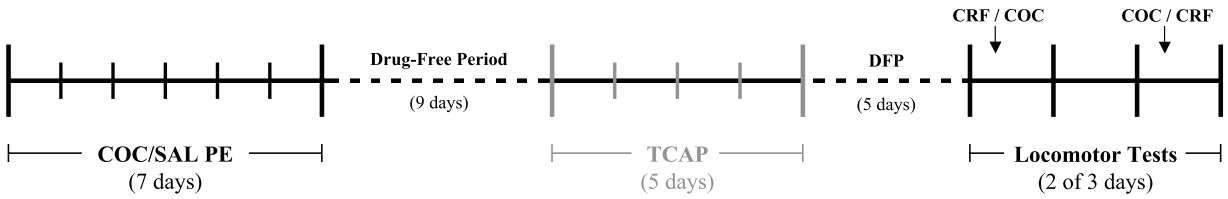
3.4.1.5.4. Phase 4: TCAP-1 Pre-Exposure

Beginning nine days into the 19-day drug-free period, rats were given five daily injections of TCAP-1 (0 or 300 pmol, i.c.v.) in their home cages.

3.4.1.5.5. Phase 5: Tests for Locomotor Sensitization

Five days after the last TCAP-1 injection, rats were given two tests for sensitization, one in response to CRF (0.5 μg; i.c.v.) and one in response to cocaine (15 mg/kg). Tests were separated by 48 h and given in a counterbalanced order. For each test, rats were initially placed in the activity chambers for 60 min. Following administration of all pretreatments and test

Figure 15



Procedural timeline for Experiment 3.

substances, rats were returned to the chambers for an additional 120 min.

3.4.2. Results

3.4.2.1. Tests for Sensitization

Distance traveled (cm) during the 60 min prior to the first test for locomotion was not altered by a prior history of cocaine or TCAP-1 pre-exposure (data not shown). Figure 16 shows the mean (±SEM) distance traveled (cm) in response to a CRF challenge (0.5 μg; i.c.v.) 19 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) and five days after the last TCAP-1 pre-exposure (0 or 300 pmol; i.c.v.). Although the test sessions were 120 min in duration, initial analyses of the data revealed that the acute activational effects of CRF occurred in the first 30 min. Thus, all subsequent analyses were based on activity monitored during this 30 min period. A two-way ANOVA revealed a significant interaction of Cocaine Pre-Exposure x TCAP-1 Pre-Exposure [$F(1,56)=7.07$, $p<.01$]. Cocaine pre-exposed rats in the 0 pmol TCAP-1 condition traveled a greater distance following the CRF challenge than their saline pre-exposed counterparts, but not those repeatedly pre-exposed to 300 pmol TCAP-1 ($p<.05$). In fact, CRF-induced activity of cocaine pre-exposed rats in the 300 pmol TCAP-1 condition did not differ from that of saline pre-exposed rats in both the 0 and 300 TCAP-1 conditions. Moreover, activity in the cocaine and TCAP-1 pre-exposed group was reduced relative to cocaine pre-exposed rats in the 0 TCAP-1 condition to a degree that approached significance ($p=0.06$). Thus, cocaine pre-exposure resulted in a sensitized locomotor response to a CRF challenge that was blocked by TCAP-1 pre-exposure.

Figure 17 shows the mean (±SEM) distance traveled (cm) in response to a cocaine challenge (15 mg/kg; i.p.) 19 days after repeated cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) and five days after repeated TCAP-1 pre-exposure (0 or 300 pmol; i.c.v.). Again, activity in the

first 30 min of the 120-min locomotor test was analyzed, when the greatest activational effects of the cocaine challenge occurred. A two-way ANOVA revealed only a main effect of Cocaine Pre-Exposure [$F(1,58)=11.17$, $p<.001$]. Cocaine pre-exposed rats traveled a greater distance following the cocaine challenge than their saline counterparts, irrespective of TCAP-1 pre-exposure. Thus, the cocaine challenge induced a sensitized locomotor response in cocaine pre-exposed rats, and this effect was not altered by TCAP-1 pre-exposure.

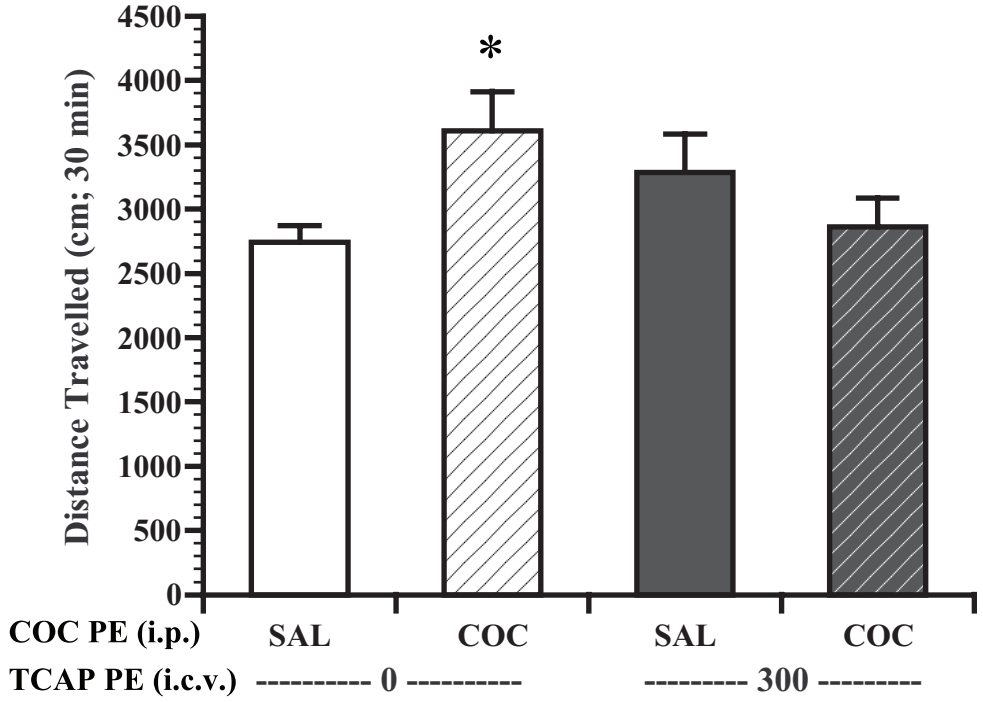
3.5. Discussion

The series of experiments presented in this chapter provide the first evidence that repeated exposure to TCAP-1 blocks the effects of CRF on the reinstatement of cocaine seeking, and on the expression of cocaine-induced behavioural sensitization. TCAP-1 was found to selectively interfere in the expression of CRF-induced behaviours in that TCAP-1 was without effect on either footshock- or cocaine-induced reinstatement of cocaine seeking, or the expression of cocaine-induced behavioural sensitization to a cocaine challenge.

3.5.1. Acute TCAP-1 Pretreatment was Without Effect on Reinstatement

In Experiment 1, acute TCAP-1 treatment was found to be without effect on reinstatement responding, when administered either alone or immediately prior to a reinstating stimulus. These null effects were not surprising given that the few reported behavioural effects of acute TCAP-1 are modest and depend to a large extent on the baseline characteristics of the subjects being tested. For example, in the EPM, acute i.c.v. treatment with TCAP-1, relative to saline, had no effect on open or closed arm time or entries (the principle measures of anxiety in this task), but increased stretch-attend postures (Tan et al., 2009). Furthermore, acute intra-BLA injections of TCAP-1 had no consistent effect on the acoustic startle reflex, but instead normalized individual differences in baseline reactivity on this measure (Wang et al., 2005b).

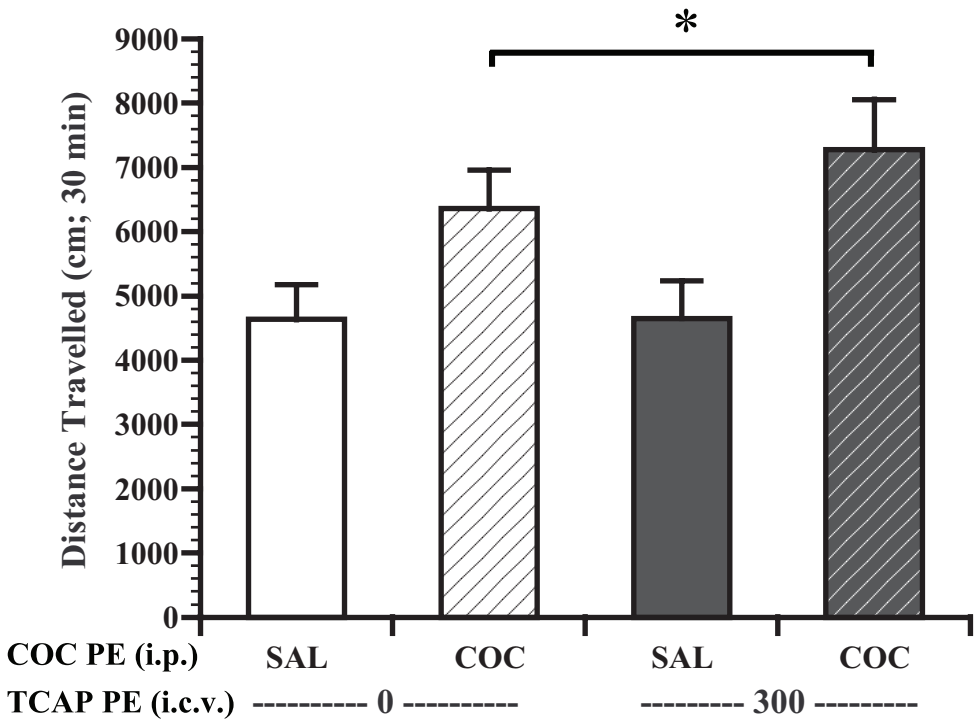
Figure 16



Mean (±SEM) distance traveled (cm) in response to a CRF challenge (0.5 μg; i.c.v.) 19 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) and five days after the last TCAP-1 pre-exposure (0 or 300 pmol; i.c.v.) [Experiment 3]. Two-way ANOVA: COC PE x TCAP-1 PE [$F(1,56)=7.07$, $p<.01$]; LSD post-hoc.

* Different from saline (SAL) condition, $p<.05$.

Figure 17



Mean (±SEM) distance traveled (cm) in response to a cocaine challenge (15 mg/kg; i.p.) 19 days after repeated cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) and five days after repeated TCAP-1 pre-exposure (0 or 300 pmol; i.c.v.) [Experiment 3]. Two-way ANOVA: Main effect of COC PE [$F(1,58)=11.17$, $p<.001$].

* Different from saline (SAL) condition, $p<.001$.

Moreover, despite evidence that acute i.c.v. pretreatment with TCAP-1 attenuates CRF-induced c-fos expression in limbic forebrain regions such as the hippocampus, amygdala, and mPFC (Tan et al., 2009), no work to date has revealed any effect of acute TCAP-1 on CRF-induced behaviour.

3.5.2. Repeated TCAP-1 Pre-exposure was Without Effect on Extinction Responding and Baseline Locomotion

In Experiments 2 and 3, the first two behaviours assessed following repeated TCAP-1 pre-exposure were extinction responding and baseline locomotion prior to testing for behavioural sensitization; TCAP-1 was without effect on both. These findings are consistent with evidence that 5-day TCAP-1 treatment is ineffective in modulating baseline anxiety-like behaviour and locomotion in the EPM and open field (Al Chawaf et al., 2007b; Tan et al., 2008; 2009). They further indicate that TCAP-1 treatment produced no long-lasting, non-specific motoric effects, and did not alter the behavioural expression of any contextual or cocaine-associated learning acquired prior to treatment. As such, the present effects of TCAP-1 appear to be selective to the reinstatement of cocaine seeking and the expression of cocaine-induced behavioural sensitization.

3.5.3. Repeated TCAP-1 Pre-exposure Selectively Blocked CRF-induced Behaviours

The findings from Experiments 2 and 3 showing that repeated exposure to TCAP-1 blocked the effects of CRF on cocaine-related behaviours are consistent with previous reports that repeated TCAP-1 inhibits CRF-induced behavioural anxiety (Al Chawaf et al., 2007b) and CRF-potentiated startle (Tan et al., 2008). Together, these findings provide support for the notion that TCAP-1 serves to attenuate the physiological actions of CRF (Lovejoy et al., 2009). In contrast, however, the same 5-day, 300 pmol, i.c.v. TCAP-1 treatment regimen that inhibited

the effects of CRF on acoustic startle (Tan et al., 2008), reinstatement (Experiment 2), and cocaine-sensitized locomotion (Experiment 3), has been shown to potentiate CRF-induced reductions in open arm time and entries in the EPM, and in center time and entries in the open field (Tan et al., 2008). Notably, the dose of CRF used by Tan and colleagues (2008) was between 2-6 times that used in the present studies (i.e. 1-3 μg vs. 0.5 μg), and is likely among many factors that account for these discrepancies. Indeed, TCAP-1 appears to interact with CRF in a manner that depends on the route and regimen of administration of both peptides, the behavioural test, and the baseline reactivity of the rats being tested.

One striking aspect of the present findings was the highly selective nature of the effects of TCAP-1 on behavioural responses to CRF. Particularly surprising was the observation that TCAP-1 completely blocked CRF-induced reinstatement of cocaine seeking, but failed to alter reinstatement by footshock stress, an effect mediated by CRF (Erb et al., 1998; 2001; Erb and Stewart, 1999; Wang et al., 2005a). In fact, a dose of TCAP-1 two to 20 times that associated with a complete blockade of CRF-induced reinstatement (i.e. 600 pmol) was without effect on reinstatement by footshock stress. Several factors likely underlie this differential regulation of CRF- and footshock-induced reinstatement, including the nature of the stressors (pharmacological vs. physical/environmental), the brain systems they engage (Imaki et al., 1993; Dunn et al., 2000), and the onset and duration of their neurochemical effects (Matsuzaki et al., 1989; Kalivas and Duffy, 1995; Galvez et al., 1996; Erb et al., 2000; de Groote et al., 2005). For example, noradrenaline has been found to play a critical role in footshock-induced reinstatement of cocaine seeking via enhanced neurotransmission within the ventral pathway originating in the lateral tegmental nuclei (Shaham et al., 2000), and subsequent activation of β1- and β2-adrenoceptors in the CeA and BNST (Leri et al., 2002). CRF-induced reinstatement of cocaine seeking, on the other hand, is unaltered when noradrenaline transmission is inhibited by systemic injections of the α2-adrenoceptor agonist, clonidine, at a dose known to block

footshock-induced reinstatement of cocaine seeking (Erb et al., 2000; Brown et al., 2009). Thus, it appears that CRF-induced reinstatement of cocaine seeking is less reliant on an intact noradrenaline system than is footshock-induced reinstatement.

Less surprising were the observed dissociations between the effects of TCAP-1 on the expression of CRF- and cocaine-induced reinstatement and behavioural sensitization. Indeed, CRF has been shown not to mediate the effects of cocaine priming on reinstatement (Erb et al., 1998), and the neurochemical systems mediating behavioural sensitization to cocaine are particularly dependent on the nature of the sensitizing regimen (e.g. Sorg and Kalivas, 1991; Sorg, 1992; Prasad et al., 1995). More importantly, perhaps, the pharmacological properties of, and neurocircuitry engaged by, cocaine and CRF bear limited resemblance (Hammer, 1995; Chadwick and Marsh, 1993).

The effects of repeated TCAP-1 on reinstatement of cocaine seeking were not only highly selective to reinstatement induced by CRF, but were also highly effective. Indeed, doses of 30 and 300 pmol TCAP-1 were equally effective in completely blocking the effect of CRF on the reinstatement of cocaine seeking. Similarly high potency has been observed in previous behavioural studies. For example, repeated i.v. exposures to 300 pmol TCAP-1, which would yield considerably lower effective brain concentrations of TCAP-1 relative to i.c.v. exposures to this same dose, are associated with reliable anxiolytic effects in the EPM and open field tests (Al Chawaf et al., 2007b). Likewise, repeated i.c.v. exposures to doses of TCAP-1 ranging from 60 to 300 pmol are anxiolytic in the acoustic startle procedure (Wang et al., 2005a; Tan et al., 2008).

3.5.4. Mechanisms of Interaction between TCAP-1 and CRF

3.5.4.1. Potential Regional and Cellular Interactions between TCAP-1 and CRF

The brain loci in which TCAP-1 and CRF interact to alter behaviour are currently unknown. However, the central distributions of TCAP-1, as well as teneurin-1 and β-DG, two proteins believed to serve as binding sites for TCAP-1, have been shown to be widespread, and particularly pronounced in limbic and frontal brain regions (Wang et al., 2005b; Torres et al., 2011; Li et al., 2006; Kenzelmann et al., 2008; Zaccaria et al., 2001; Ohysuka-Tsurumi et al., 2004). TCAP-1 has also been shown to attenuate CRF-induced increases in c-fos expression in similar regions, including the amygdala (Tan et al., 2009), which have been implicated in the effects of CRF on the reinstatement of cocaine seeking (Erb et al., 2001) and the expression of cocaine-induced behavioural sensitization (Erb et al., 2005). Although, unlike in the amygdala, acute TCAP-1 was without effect on CRF-induced c-fos expression in the BNST (Tan et al., 2009), repeated TCAP-1 may act in the BNST to counteract the behavioural effects of CRF. Indeed, intra-BNST injections of CRF induce the reinstatement of cocaine seeking (Erb and Stewart, 1999), as well as several other stress-related behaviours (e.g. Lee and Davis, 1997; Sahuque et al., 2006; Lee et al., 2008). Moreover, excitotoxic lesions of the BNST, and intra-BNST infusion of the non-selective CRF receptor antagonist, α-helical CRF_{9-41}, like repeated i.c.v. treatment with TCAP-1 (Tan et al., 2008), block the enhanced acoustic startle response seen in rats following i.c.v. CRF administration (Lee and Davis, 1997).

The mechanisms by which TCAP-1 and CRF interact at the cellular level also remain unclear. Importantly, the TCAP-1 receptor has yet to be identified, although current evidence suggests that TCAP-1 is internalized into neurons via calveoli-mediated endocytosis following a putative interaction with teneurin-1 or β-DG (Chand et al., 2011). It is clear, however, that TCAP-1 does not act directly on CRF receptors to mediate its effects. Indeed, TCAP-1 has been shown in cell culture to act independently of CRF receptors to modulate cAMP (Wang et al.,

2005b), activate CREB and ATF1, and downregulate AP-1 (De Almeida et al., 2011; Nock et al., unpublished). TCAP-1 treatment does, however, modulate many intracellular systems recruited by CRF, including cAMP, CREB, and ERK (Grammatopoulos and Chrousos, 2002; Stern et al., 2011). In fact, it is presumably through its known ERK-mediated reorganization of the actin and tubulin cytoskeletons (Al Chawaf et al., 2007a; Chand et al., 2011) that TCAP-1 treatment increases neurite and axonal outgrowth *in vitro* (Al Chawaf et al., 2007a), and modulates dendritic branching and spine density *in vivo* (Tan et al., 2011). Interestingly, CRF has also been shown to influence cell signaling and structure through ERK- and actin-dependent regulation of neurite outgrowth (Cibelli et al., 2001), dendritic branching (Swinny et al., 2004; Swinny and Valentino, 2006), and spine density (Chen et al., 2008). Taken together, these findings suggest that TCAP-1 may act downstream of CRF receptor activation in a manner that, through repeated exposure, induces long-lasting changes in cell signaling and structure that serve to inhibit certain cellular and behavioural effects of CRF.

3.5.4.2. Role of Cocaine Experience in the Effects of TCAP-1 on CRF-induced Behaviours

Without first knowing the mechanism by which TCAP-1 and CRF interact to alter behaviour, it is difficult to speculate on how a history of cocaine exposure might serve to change the nature of this interaction. One possibility is that TCAP-1 acts to normalize signaling within CRF receptor systems that have become dysregulated as a consequence of cocaine exposure. Indeed, there is considerable evidence that neuronal and behavioural responses to CRF change as a consequence of prior cocaine exposure, and that this change may involve alterations in CRF receptor affinity and intracellular signaling (Corominas et al., 2010). For example, it has been shown that central CRF injections selectively enhance c-fos mRNA expression in the CeA of animals pre-exposed to cocaine, relative to saline (Erb et al., 2005). It has also been shown that augmented CRF-induced long-term potentiation in the CeA, observed following withdrawal

from repeated cocaine administration, is mediated by enhancements in CRF_1 receptor function and signaling through the second messenger, protein kinase A (Pollandt et al., 2006). Finally, exposure to cocaine has been shown to sensitize glutamate and DA release in the VTA in response to CRF, alterations known to play a critical role in the effects of stress on the reinstatement of cocaine seeking (Wang et al., 2005a; 2007). Thus, it is conceivable that TCAP-1 interferes in CRF-induced reinstatement and CRF-induced expression of behavioural sensitization by acting to normalize changes in CRF receptor transmission.

It is well established that many *in vivo* effects of TCAP-1 are critically influenced by individual differences in the baseline reactivity of the subjects being tested. For example, TCAP-1 infusions into the BLA reduced the acoustic startle response of rats that displayed high emotionality prior to test (i.e. above average baseline startle amplitude), and enhanced the startle response of those that displayed low baseline emotionality (Wang et al., 2005b). Furthermore, i.c.v. TCAP-1 treatment has been shown to increase the coefficient of variation of neuronal c-fos expression seen in various brain regions in response to an i.c.v. CRF challenge (Tan et al., unpublished). In contrast, the present findings show that TCAP-1 robustly inhibits the effects of CRF on cocaine-related behaviours with very low statistical variation. Given that cocaine is a potent psychostimulant capable of inducing profound neuronal and behavioural plasticity, including the persistent enhancement of emotionality (e.g. Gordon and Rosen, 1999; Erb et al., 2006b), it is possible that a history of cocaine exposure may serve to equalize and even enhance the baseline responsivity of all subjects, thereby biasing the effects of TCAP-1 on CRF-induced, cocaine-related behaviours towards reliable inhibition.

3.5.4.3. Potential Role for CREB and BDNF in the Effects of TCAP-1 on CRF-induced Behaviours

One intracellular system that may help mediate the effects of TCAP-1 on CRF-induced, cocaine-related behaviour is CREB. In addition to being implicated in the cellular response to both TCAP-1 and CRF (De Almeida et al., 2011; Stern et al., 2011), CREB has been shown to play an important, and even selective, role in the effects of stress on cocaine-related behaviour. For example, Blendy and colleagues showed that the reinstatement of extinguished cocaine CPP by exposure to forced swim stress, but not a cocaine challenge, was impaired in CREB-deficient mice (Kreibich and Blendy, 2004). Furthermore, cocaine-conditioned mice, relative to saline-exposed mice, exhibited potentiated levels of phosphorylated CREB in the amygdala in response to swim stress, but not a cocaine challenge (Kreibich and Blendy, 2004). Consistent with evidence of a critical role for the BNST and VTA in stress-induced reinstatement behaviours (Erb and Stewart, 1999; Wang et al., 2005a), CREB-deficient mice, relative to wild-type, also showed blunted activation of BNST efferents to the VTA, as assessed by c-fos expression, in response to forced swim stress (Briand et al., 2010). CREB activation has also been implicated in the cross-sensitization between stress and cocaine. For example, chronic exposure to forced swim stress in advance of conditioning enhanced the acquisition of cocaine CPP in wild-type, but not CREB-deficient mice (Kreibich et al., 2009). Interestingly, pretreatment with the CRF_1 receptor antagonist, antalarmin, prior to forced swim exposure similarly blocked the stress-enhanced acquisition of cocaine CPP (Kreibich et al., 2009). Collectively, these results suggest that CREB, through its involvement in cocaine-related, CRF-mediated behavioural responses to stress, may be an intracellular system through which TCAP-1 can modulate the effects of CRF on reinstatement and behavioural sensitization.

Another system potentially involved in the effects of TCAP-1 on cocaine-related behaviour is that of the neurotrophin, BDNF. Shown to be negatively regulated *in vitro* by

TCAP-1 (Ng et al., 2010), BDNF has also been implicated in the expression of both the reinstatement of cocaine seeking and cocaine-induced behavioural sensitization. For example, Shaham and colleagues demonstrated that a single infusion of BDNF into the VTA 1-2 h following the final session of cocaine self-administration potentiated subsequent cue-induced reinstatement of cocaine seeking when tested up to 30 days following cocaine withdrawal (Lu et al., 2004). Similarly, 5 daily infusions of BDNF into the NAc during cocaine self-administration training increased subsequent reinstatement of cocaine-seeking behaviour by cocaine, cocaine-associated cues, or footshock stress; on the other hand, intra-NAc infusions of a BDNF antibody attenuated reinstatement by each of these stimuli (Graham et al., 2007). Opposite to its effects in the VTA and NAc, BDNF administration into the dorsomedial PFC immediately following the last of 10 cocaine self-administration sessions attenuated cocaine- and cue-induced reinstatement of cocaine seeking when tested 6 days later (Berglind et al., 2007). Moreover, in the mPFC, bilateral downregulation of the high affinity BDNF receptor, tropomyosin receptor kinase B (TrkB), attenuated the cocaine-sensitized locomotor response to a cocaine challenge (Lu et al., 2010).

The effects of BDNF on cocaine-related behaviours are neither selective to those induced by stress, nor are there any data suggesting that such effects are CRF receptor-dependent. Thus, it is presently unclear how TCAP-1 regulation of BDNF signaling could account for the ability of TCAP-1 to selectively interfere with CRF-induced behaviours. However, through their many converging intracellular systems (e.g. ERK, CREB; Patapoutian and Reichardt, 2001; Grammatopoulos and Chrousos, 2002), BDNF and CRF have been shown to interact at both the cellular and behavioural level. In fact, BDNF modulates various cocaine-related behaviours through its recruitment of ERK and CREB signaling (Lu et al., 2004; Fumagalli et al., 2009; Whitfield et al., 2011), the same intracellular systems that mediate BDNF-CRF interactions. For example, CRF_1 receptor signaling upregulated BDNF mRNA

through a cAMP-PKA-CREB-dependent mechanism in cerebellar granular cells (Bayatti et al., 2005), and potentiated TrkB signaling in locus coeruleus neurons through recruitment of ERK (Traver et al., 2006). Furthermore, CRF infusion into the rat dentate gyrus dose-dependently increased local BDNF mRNA expression (Ma et al., 1999). Functionally, intra-dentate infusions of CRF improved memory retention in an inhibitory avoidance learning task, and this improvement was blocked by pretreatment with a BDNF antisense oligonucleotide (Ma et al., 1999). Therefore, given the role for BDNF in cocaine-related behaviours, and the evidence that CRF can positively regulate BDNF activity, it is possible that the negative regulation of BDNF by TCAP-1 contributes to its ability to interfere with the effects of CRF on cocaine-related behaviours.

CHAPTER 4

Role of Endocannabinoid CB_1 Receptors in the Reinstatement of Cocaine Seeking and Cocaine Sensitization

CHAPTER 4

Role of Endocannabinoid CB_1 Receptors in the Reinstatement of Cocaine Seeking and Cocaine Sensitization

4.1. Introduction

Extending on a growing body of work exploring interactions between CRF and other stress-related neurochemical systems in the regulation of cocaine-related behaviours, the experiments presented in Chapter 3 represent the first investigation of a role for the novel neuropeptide, TCAP-1, in the actions of CRF on the reinstatement of cocaine seeking, and the expression of cocaine-induced behavioural sensitization. In this chapter, a further extension of the neurobiological characterization of CRF in cocaine-related behaviours is provided in a series of experiments that parallel those presented in Chapter 3, but with a focus on possible functional interactions between eCB CB_1 receptors and CRF. As described in Chapter 1, such a parallel consideration of TCAP-1 and eCB systems is justified on the basis of their considerable overlap in neuroanatomical distribution, their common influence on many cellular processes and behaviours, and their notable neuroanatomical and functional interactions with CRF (see Chapter 1).

As previously described, the eCBs comprise a family of retrograde signaling molecules that act presynaptically on CB_1 receptors to suppress the release of a variety of neurochemicals (Lovinger, 2008). CB_1 receptors are densely expressed in brain regions associated with emotional regulation, such as the amygdala, PFC, hippocampus, hypothalamus, and striatum (Herkenham et al., 1990; 1991; Tsou et al., 1998; Moldrich and Wenger, 2000). Consistent with this pattern of expression, the eCB system has been implicated in a variety of behavioural effects of drugs of abuse and stress, including those unique to subjects with previous drug exposure.

Although a growing number of studies have investigated the role of CB_1 receptors in the

expression of various long-term behavioural effects of cocaine, including the reinstatement of cocaine seeking and the expression of cocaine-induced behavioural sensitization, some of this work has yielded inconsistent findings. For example, several reports have shown that systemic pretreatment with CB_1 receptor antagonists, such as SR141716, blocked the reinstatement of cocaine seeking induced by non-contingent administration of cocaine (De Vries et al., 2001; Filip et al., 2006a; Xi et al., 2006; but see Ward et al., 2009); one such study further reported that SR141716 was without effect on reinstatement induced by footshock stress (De Vries et al., 2001). On the other hand, in a recent study using the CPP procedure, somewhat opposing findings were obtained, whereby pretreatment with the selective CB_1 receptor antagonist, AM251, was without effect on cocaine-induced reinstatement of an extinguished cocaine CPP, but blocked its reinstatement by forced swim stress (Vaughn et al., 2011). Although the role of CB_1 receptors in cocaine-induced behavioural sensitization has been the subject of fewer investigations, the studies that have been carried out to date indicate an antagonizing effect of acute administration of SR141716 on cocaine-induced locomotor sensitization (Filip et al., 2006a; Ramiro-Fuentes and Fernandez-Espejo, 2011).

As detailed in Chapter 1, considerable neuroanatomical and neuroendocrine evidence indicates that the eCB system is capable of regulating the actions of CRF. Briefly, *in situ* hybridization studies reveal significant colocalization between the mRNA of CB_1 receptors and both CRF peptide and CRF_1 receptors in numerous stress-related brain regions (Hermann and Lutz, 2005; Cota et al., 2007). Moreover, eCB signaling in the PVN, BLA, and PFC has been shown to critically influence the activity of CRF-secreting neurons in the PVN (Di et al., 2003; Evanson et al., 2010; Hill et al., 2009; Hill et al., 2011). Despite such evidence, there remains a paucity of data addressing direct functional interactions between eCBs and CRF in the regulation of behavioural responses, including cocaine-related responses.

Accordingly, in a manner that parallels the work presented in Chapter 3, the experiments

presented in this chapter (Experiments 4-6) were designed to explore a role for CB_1 receptors in the reinstatement of cocaine seeking and the expression of cocaine-induced behavioural sensitization. In part, the work was designed to confirm previously published findings on the effects of CB_1 receptor manipulations on reinstatement of cocaine seeking and the expression of behavioural sensitization to cocaine. The primary and novel objective of this work, however, was to determine the effects of CB_1 receptor manipulation on the reinstatement of cocaine seeking induced by CRF, and on the expression of cocaine-induced behavioural sensitization elicited by a CRF challenge. This was accomplished via acute and central administration of the selective CB_1 receptor antagonist, AM251, prior to tests for reinstatement and sensitization. First, in Experiment 4, the effects of AM251 on the reinstatement of cocaine seeking induced by i.c.v. injections of CRF, exposure to footshock stress, and priming i.p. injections of cocaine were assessed. To ensure that any attenuation of lever pressing observed in Experiment 4 could not be attributed to non-specific motoric effects of AM251, Experiment 5 was conducted to assess the effects of AM251 on sucrose self-administration. Lastly, Experiment 6 tested the effects of AM251 on the expression of cocaine-induced behavioural sensitization to challenge injections of CRF and cocaine. AM251 was found to interfere in the expression of CRF-, but not footshock- or cocaine-induced reinstatement of cocaine seeking, as well as in the expression of cocaine-induced behavioural sensitization to challenge injections of both CRF and cocaine.

4.2. Experiment 4: Effects of AM251 on Reinstatement of Cocaine Seeking

4.2.1. Materials and Methods

4.2.1.1. Subjects

A total of 67 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

4.2.1.2. Surgery

Rats were surgically implanted with both an i.v. catheter and i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2).

4.2.1.3. Apparatus

All behavioural procedures were carried out in the drug self-administration chambers (Med Associates) described in Chapter 2 (Section 2.5.1).

4.2.1.4. Drugs

Cocaine HCl (Medisca Pharmaceuticals) and CRF (Sigma-Aldrich) were both dissolved in sterile, physiological saline. AM251 (Tocris Bioscience) was dissolved in DMSO (Sigma-Aldrich). The dose range and vehicle used for AM251 was based on previous work (Sink et al., 2009). Central injections of CRF and AM251 were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

4.2.1.5. Reinstatement Procedures

Figure 18 shows the procedural timeline of Experiment 4.

4.2.1.5.1. Phase 1: Self-Administration

Rats were trained to self-administer cocaine (0.23 mg/65 µl infusion, i.v.) for a total of 8-10 days using the procedures described in Chapter 2 (Section 2.6.1.1).

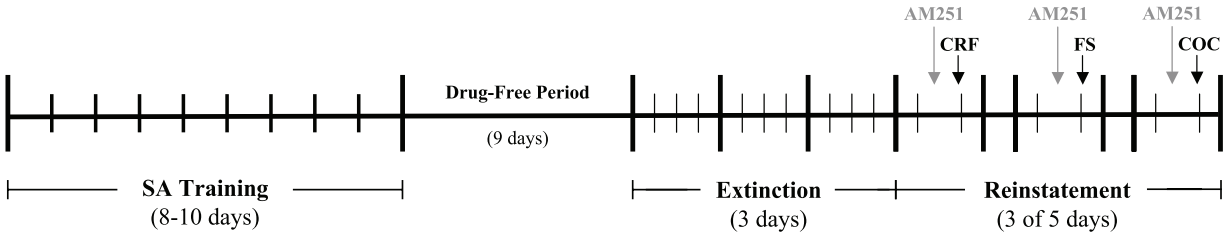
4.2.1.5.2. Phase 2: Drug-Free Period

Rats were maintained in their home cages for nine days under the vivarium conditions described in Chapter 2 (Section 2.6.1.2).

4.2.1.5.3. Phase 3: Extinction

Extinction Days 1-3 were carried out as described in Chapter 2 (Section 2.6.1.3).

Figure 18



Procedural timeline for Experiment 4.

4.2.1.5.4. Phase 4: Tests for Reinstatement

Following extinction, rats were tested for CRF-, footshock- and cocaine-induced reinstatement, as described in Chapter 2 (Section 2.6.1.4). At the start of each test day, rats were given three 60-min extinction sessions. Subsequently, rats were given an injection of AM251 (0, 10, 100, or 200 μg; i.c.v.), 30 min prior to exposure to a reinstatement trigger [CRF (0.5 μg; i.c.v.), Footshock (20 min; 0.9 mA), Cocaine (10 mg/kg; i.p.)] or its corresponding baseline condition [i.c.v. saline [VEH], No Footshock [No FS], i.p. saline [VEH]]. Thirty, 0, or 10 min following the CRF, footshock, or cocaine challenges, respectively, tests for reinstatement began, whereby the previously drug-reinforced lever was extended into the chamber and responding was recorded over a 60-min period. Different groups of rats were pretreated with different doses of AM251. Each rat was given up to 3 tests for reinstatement (one of these tests was a baseline test) on 3 separate days; tests were separated by 48 h, and were administered in a counterbalanced order. Responding on the active lever following exposure to CRF, footshock, or cocaine, relative to the baseline condition, was used as a measure of the reinstatement of cocaine seeking.

4.2.2. Results

4.2.2.1. Self-Administration

Once rats acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period. The mean ± SEM number of infusions made during the 180-min sessions on the last two days of training was 38.19 ± 1.61 and 38.74 ± 1.60, respectively, corresponding to average daily cocaine intake of 8.78 ± 0.37 and 8.91 ± 0.37 mg.

4.2.2.2. Extinction

At the start of the extinction phase, rats showed characteristic heightened responding on the previously drug-reinforced lever, averaging 86.70 ± 6.48 in the first 60-min extinction session. This rate of responding gradually declined to 6.27 ± 1.12 active lever responses in the final 60-min session on Day 3 of extinction.

4.2.2.3. Tests for Reinstatement

Figure 19 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 10, 100, or 200 μg; i.c.v.) and subsequently challenged with saline (VEH; i.c.v.) or CRF (0.5 μg; i.c.v.). It can be seen that, across the range of doses tested, AM251 pretreatment blocked CRF-induced reinstatement of cocaine seeking. Indeed, a repeated measures ANOVA of total responses on the active lever revealed significant main effects of CRF [$F(1,55)=8.59$, $p<.005$] and AM251 [$F(3,55)=5.62$, $p<.005$], and a significant CRF x AM251 interaction [$F(3,55)=7.30$, $p<.001$]. In the 0 μg AM251 group, CRF, relative to VEH, induced a higher level of responding on the active lever ($p<.005$); in each of the 10, 100, and 200 μg AM251 groups, this difference between CRF and VEH tests was non-significant. In addition, rats in the 0 μg AM251 group, relative to other dose groups, responded significantly more on the active lever following CRF treatment (p's<.01). Finally, a repeated measures ANOVA of inactive lever responding revealed no significant effects. Indeed, responding on the inactive lever was very low (less than 25% of active lever responding) across all pretreatment and test conditions.

Figure 20 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 100, or 200 μg; i.c.v.) and subsequently exposed to intermittent footshock (20 min; 0.9 mA) or no footshock. In this case, a repeated measures ANOVA of responses on the active

lever revealed only a significant main effect of Footshock [$F(1,25)=9.24$, $p<.005$]. Indeed, irrespective of the dose of AM251 administered, rats responded significantly more under the footshock than no footshock test condition. Thus, whereas AM251 pretreatment blocked the effect of i.c.v. CRF on reinstatement of cocaine seeking, it was without effect on reinstatement induced by footshock stress. Again, repeated measures ANOVA of inactive lever responding revealed no significant effects. Indeed, responding on the inactive lever was very low (less than 25% of active lever responding) across all pretreatment and test conditions.

Figure 21 shows the mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 100, or 200 μg; i.c.v.) and subsequently challenged with saline (VEH; i.p.) or cocaine (15 mg/kg; i.p.). Similar to the footshock condition, a repeated measures ANOVA of active lever responding revealed only a significant main effect of Cocaine [$F(1,33)=32.56$, $p<.001$]. Again, irrespective of AM251 pretreatment, cocaine induced a higher level of responding on the active lever relative to that seen following VEH. Thus, AM251 pretreatment had no effect on cocaine-induced reinstatement. A repeated measures ANOVA of inactive lever responding revealed no significant effects. Indeed, responding on the inactive lever was very low (less than 20% of active lever responding) across all pretreatment and test conditions.

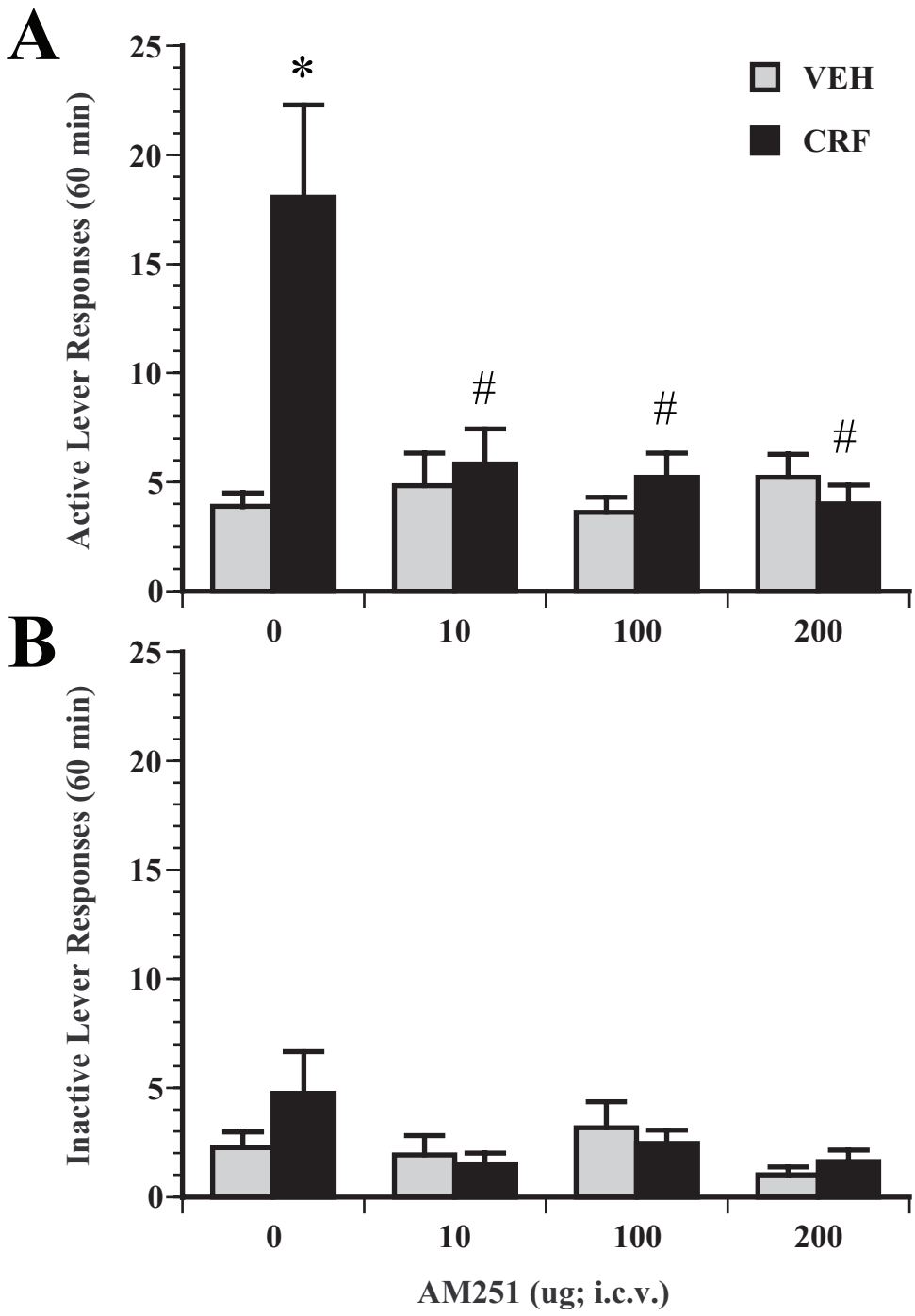
4.3. Experiment 5: Effects of AM251 on Sucrose Self-Administration

4.3.1. Materials and Methods

4.3.1.1. Subjects

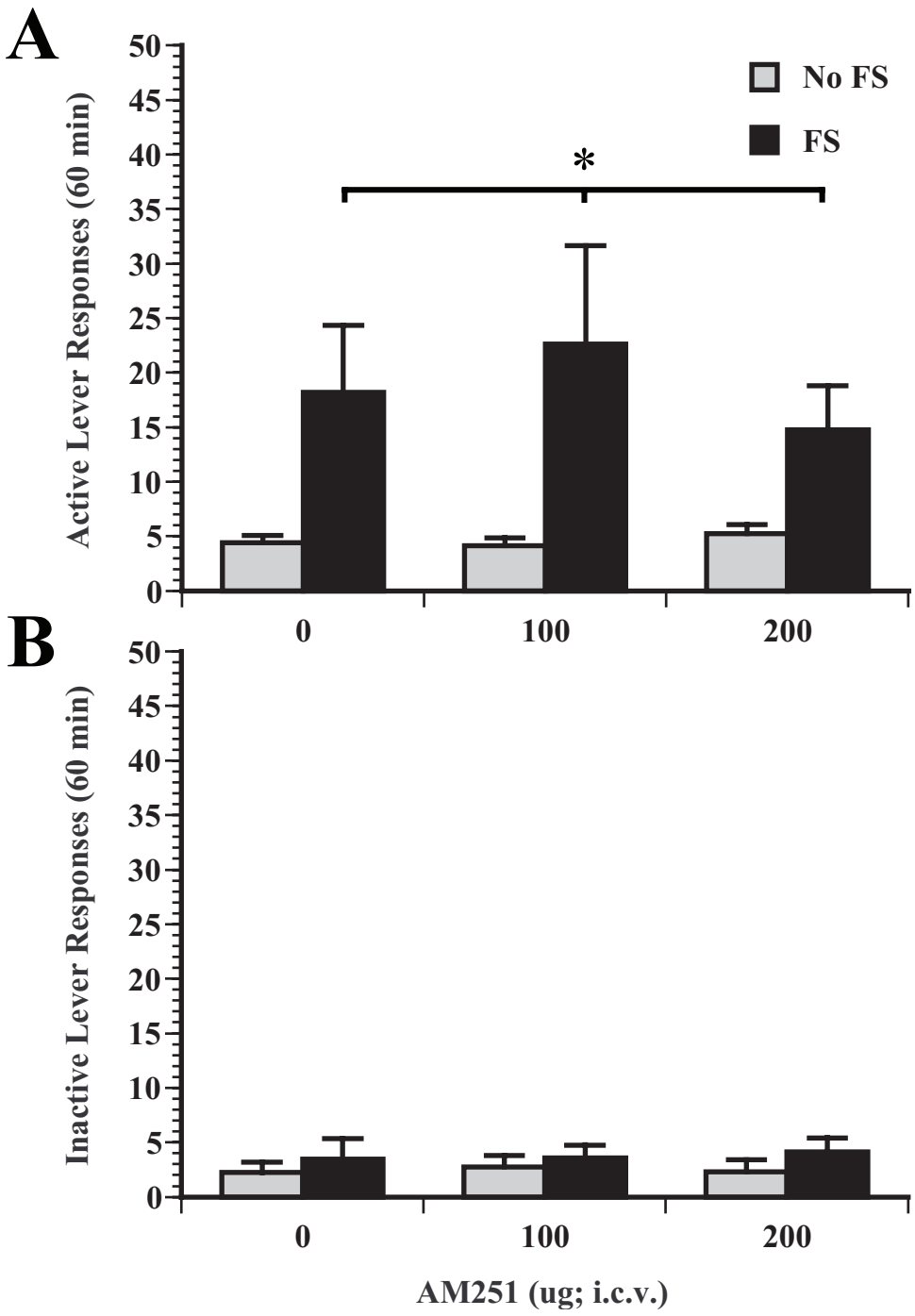
A total of 8 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

Figure 19



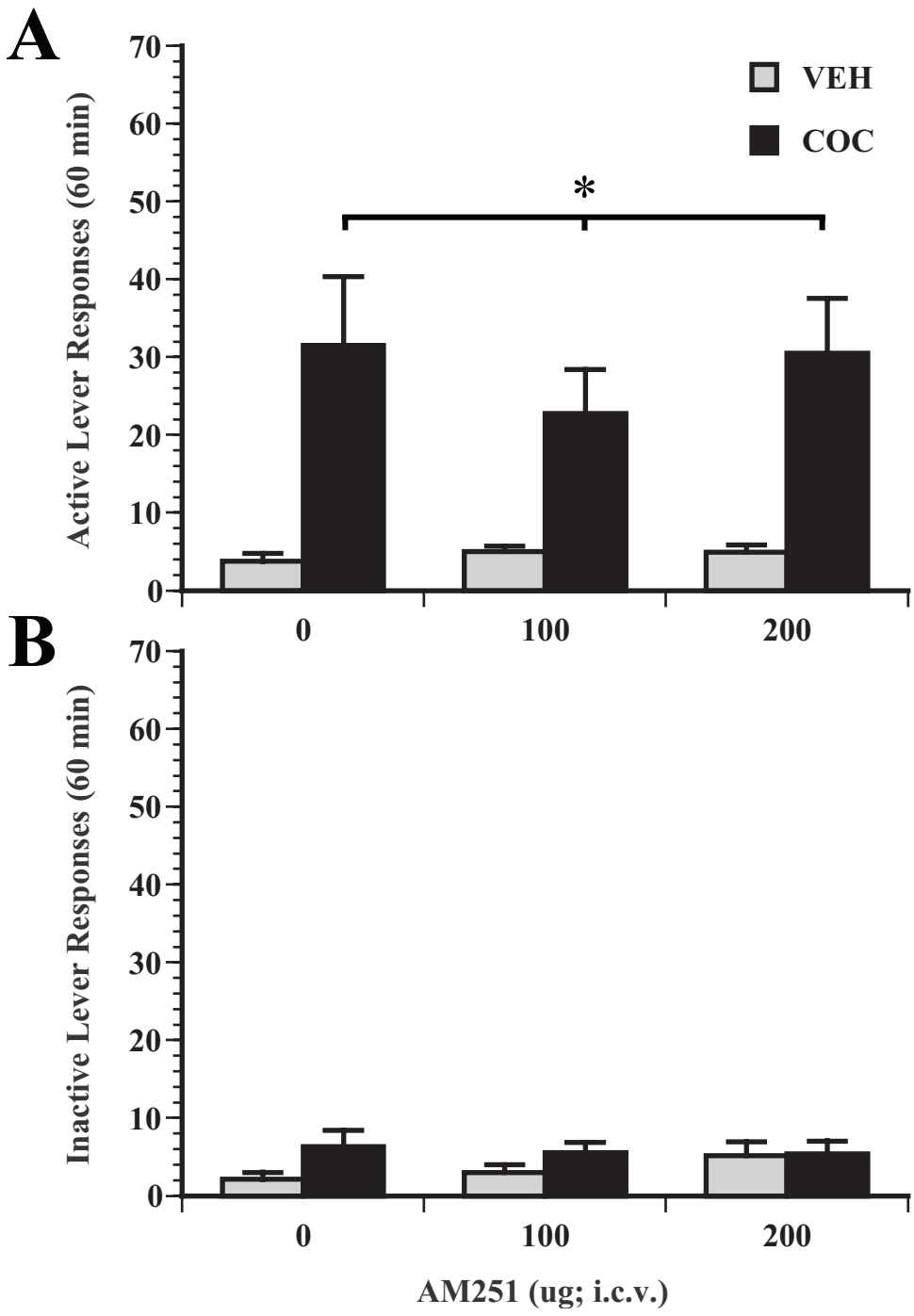
Mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 10, 100, or 200 µg; i.c.v.) and subsequently challenged with saline (VEH; i.c.v.) or CRF (0.5 µg; i.c.v.) [Experiment 4]. (A) Repeated measures ANOVA: Main effects of CRF [$F(1,55)=8.59$, $p<.005$], AM251 [$F(3,55)=5.62$, $p<.005$], CRF x AM251 interaction [$F(3,55)=7.30$, $p<.001$]; LSD post-hoc. * Different from VEH condition, $p<.005$; # Different from 0 µg AM251 condition, $p<.01$.

Figure 20



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 100, or 200 µg; i.c.v.) and subsequently exposed to intermittent footshock (FS; 20 min; 0.9 mA) or no footshock (No FS) [Experiment 4]. (A) Repeated measures ANOVA: Main effect of FS [$F(1,25)=9.24$, $p<.005$]. * Different from No FS condition, $p<.005$.

Figure 21



Mean (±SEM) number of responses on the active (A) and inactive (B) levers during 60-min reinstatement test sessions prior to which rats were pretreated with AM251 (0, 100, or 200 µg; i.c.v.) and subsequently challenged with saline (VEH; i.p.) or cocaine (15 mg/kg; i.p.) [Experiment 4]. (A) Repeated measures ANOVA: Main effect of COC [$F(1,33)=32.56$, $p<.001$]. * Different from VEH condition, $p<.001$.

4.3.1.2. Surgery

Rats were surgically implanted with an i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2.1).

4.3.1.3. Apparatus

All behavioural procedures were carried out in the sucrose self-administration chambers (Med Associates) described in Chapter 2 (Section 2.5.2).

4.3.1.4. Drugs

AM251 (Tocris Bioscience) was dissolved in DMSO (Sigma-Aldrich). The dose range and vehicle used for AM251 was based on the results of Experiment 5, as well as previous work (Sink et al., 2009). Central injections of AM251 were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

4.3.1.5. Procedures

Figure 22 shows the procedural timeline of Experiment 5.

4.3.1.5.1. Training for Sucrose Self-Administration

Rats were trained in daily 180-min sessions to lever press for sucrose pellets (45 mg; Bio-Serv) on a FR-1 schedule of reinforcement for 8-10 days. Upon reaching a stable rate of sucrose reinforcement (<10% variability in two consecutive sessions), and following a minimum of seven training sessions, rats were given a mock i.c.v. injection 30 min prior to the next training session to acclimatize them to manipulations that occurred in the subsequent test phase.

4.3.1.5.2. Effects of AM251 on Sucrose Self-administration

Tests for non-specific motoric effects of AM251 on lever pressing for sucrose pellets were conducted on three of the five sessions following training. Thirty min prior to each 180-min test session, rats was injected with a dose of AM251 (0, 100, and 200 µg; i.c.v.); all rats

were tested at each dose, on separate test days in a counterbalanced order. Test sessions were separated by 48 h, with intervening maintenance self-administration sessions.

4.3.2. Results

Figure 23 shows the mean (±SEM) number of reinforced and total responses on the active (A) and inactive (B) levers during 180-min sucrose self-administration sessions following administration of AM251 (0, 100, and 200 μg; i.c.v.). It can be seen that AM251 had no effect on responding for sucrose pellets, at any dose. Repeated measures ANOVAs of each measure revealed no significant main effects of AM251 Pretreatment, indicating no non-specific motoric effects of the antagonist.

4.4. Experiment 6: Effects of AM251 on Cocaine Sensitization

4.4.1. Materials and Methods

4.4.1.1. Subjects

A total of 55 male Wistar rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

4.4.1.2. Surgery

Rats were surgically implanted with an i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2.1).

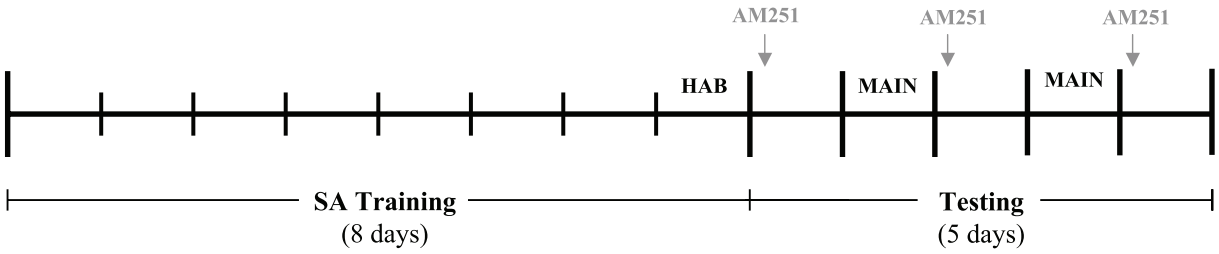
4.4.1.3. Apparatus

All behavioural procedures were carried out in the locomotor testing apparatus described in Chapter 2 (Section 2.5.3).

4.4.1.4. Drugs

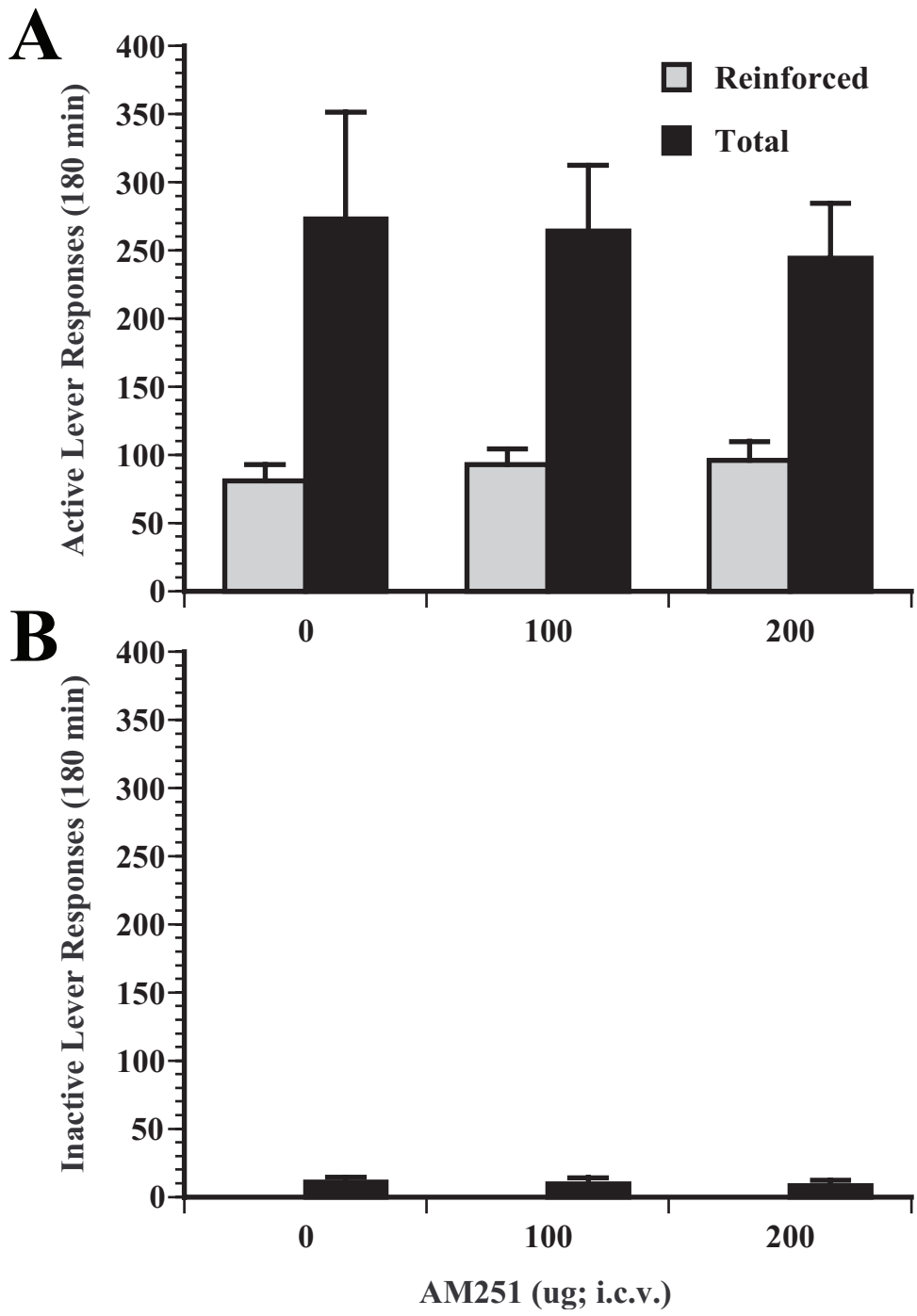
Cocaine HCl (Medisca Pharmaceuticals) and CRF (Sigma-Aldrich) were dissolved in sterile, physiological saline. AM251 was dissolved in DMSO (Tocris Bioscience). The dose

Figure 22



Procedural timeline for Experiment 5.

Figure 23



Mean (±SEM) number of reinforced and total responses on the active (A) and inactive (B) levers during 180-min sucrose self-administration sessions following administration of AM251 (0, 100, and 200 μg; i.c.v.) [Experiment 5].

range and vehicle used for AM251 was based on the results of Experiment 4, as well as previous work (Sink et al., 2009). Central injections of CRF and AM251 were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

4.4.1.5. Sensitization Procedures

Figure 24 shows the procedural timeline of Experiment 6.

4.4.1.5.1. Phase 1: Habituation

Rats were transported to the locomotor testing room, injected with saline (i.p.), and assessed for basal activity levels, as described in Chapter 2 (Section 2.6.3.1).

4.4.1.5.2. Phase 2: Cocaine Pre-Exposure

Starting 48 h after habituation, rats were given once daily injections of cocaine or saline for seven days. The first and last injections (0 or 15 mg/kg, i.p.) were administered in the activity chambers, immediately after a 60-min habituation period. The five intervening injections were given in the home cages (0 or 30 mg/kg, i.p.).

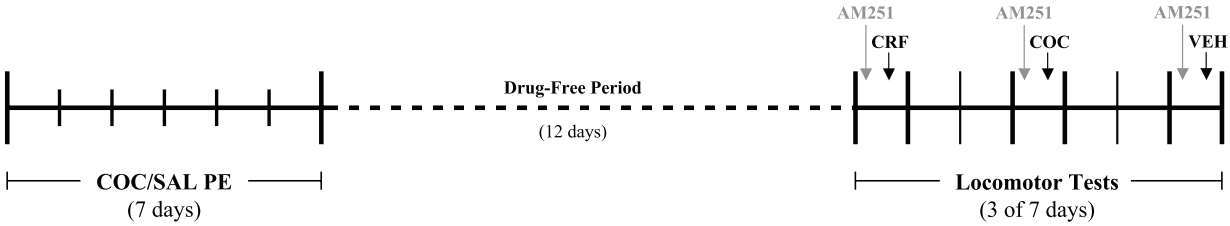
4.4.1.5.3. Phase 3: Drug-Free Period

Rats were maintained in their home cages for 12 days under the vivarium conditions described in Chapter 2 (Section 2.6.3.3).

4.4.1.5.4. Phase 4: Tests for Locomotor Sensitization

Following the drug-free period, rats were given three tests for sensitization, one in response to each of CRF (0.5 µg; i.c.v.), cocaine (15 mg/kg; i.p.), and saline (i.c.v.). Tests were separated by 48 h and given in a counterbalanced order. For each test, rats were initially placed in the activity chambers for 60 min. Following pretreatment with AM251 and administration of the test challenge, rats were returned to the chambers for an additional 120 min.

Figure 24



Procedural timeline for Experiment 6.

4.4.2. Results

4.4.2.1. Tests for Sensitization

Figure 25 shows the mean (±SEM) distance traveled (cm) in response to a CRF challenge (0.5 μg; i.c.v.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 μg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p). Although the test sessions were 120 min in duration, initial analyses of the data revealed that the acute activational effects of CRF occurred in the first 60 min. Thus, all subsequent analyses were based on activity monitored during this first hour of testing. A two-way ANOVA of distance travelled in the first 60 min revealed a significant interaction of Cocaine Pre-Exposure x AM251 Pretreatment [$F(2,48)=3.36$, $p<.05$]. Subsequent analyses of this interaction revealed that in rats pretreated with 0 μg AM251, CRF induced a sensitized locomotor response in those pre-exposed to cocaine relative to saline; however, pretreatment with 10 or 100 μg AM251 blocked this effect of CRF (p's<.05). Moreover, in cocaine pre-exposed rats, 10 and 100 μg AM251, relative to 0 μg AM251, reduced CRF-induced activity (p's<.05). Thus, cocaine pre-exposure resulted in a sensitized response to a CRF challenge that was blocked by pretreatment with AM251.

Figure 26 shows the mean (±SEM) distance traveled (cm) in response to a cocaine challenge (15 mg/kg; i.p.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 μg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.). Here, the first 30 min of the 120-min locomotor test were analyzed, corresponding to the time interval in which the greatest activational effects of the cocaine challenge were observed. Analyses of these data revealed a pattern of results that were very similar to those just described for the CRF test condition. A two-way ANOVA of distance travelled in the first 30 min revealed a significant interaction of Cocaine Pre-Exposure x AM251 Pretreatment [$F(2,49)=3.27$, $p<.05$].

Subsequent analyses of this interaction revealed that in rats pretreated with 0 μg AM251, the cocaine challenge induced a sensitized locomotor response in those pre-exposed to cocaine relative to saline; however, pretreatment with 10 or 100 μg AM251 blocked this effect (p's<.05). Moreover, in cocaine pre-exposed rats, 10 and 100 μg AM251, relative to 0 μg AM251, reduced cocaine-induced activity (p's<.05). Thus, cocaine pre-exposure resulted in a sensitized response to a cocaine challenge that was blocked by pretreatment with AM251.

Figure 27 shows the mean (±SEM) distance traveled (cm) in response to a saline challenge (i.c.v.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 μg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.). Although the data for the first 30 min of the test session are presented in Figure 27, two-way ANOVA for either the first 30 or 60 min of the session revealed no significant main effects or interactions of Cocaine Pre-Exposure and AM251 Pretreatment. Thus, AM251 alone did not alter locomotor activity, nor did it differentially alter activity in cocaine, relative to saline, pre-exposed rats.

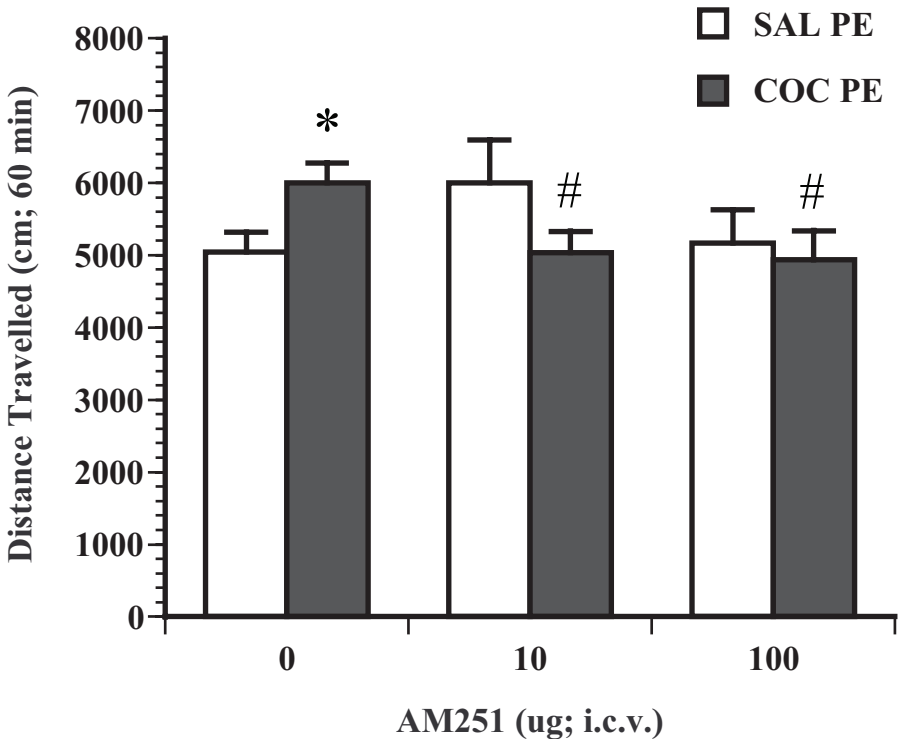
4.5. Discussion

The series of experiments presented in this chapter provide the first evidence of a role for CB_1 receptor transmission in the effects of CRF on cocaine-related behaviours. Specifically, central administration of the CB_1 receptor antagonist, AM251, blocked the reinstatement of cocaine seeking induced by CRF, but was without effect on reinstatement induced by footshock or cocaine. In contrast, central administration of AM251 blocked the expression of behavioural sensitization in response to both CRF and cocaine challenges.

4.5.1. AM251 Selectively Blocked CRF-induced Reinstatement of Cocaine Seeking

In Experiment 4, AM251 pretreatment completely blocked CRF-induced reinstatement of cocaine seeking, but failed to alter reinstatement by footshock stress. The null effect of

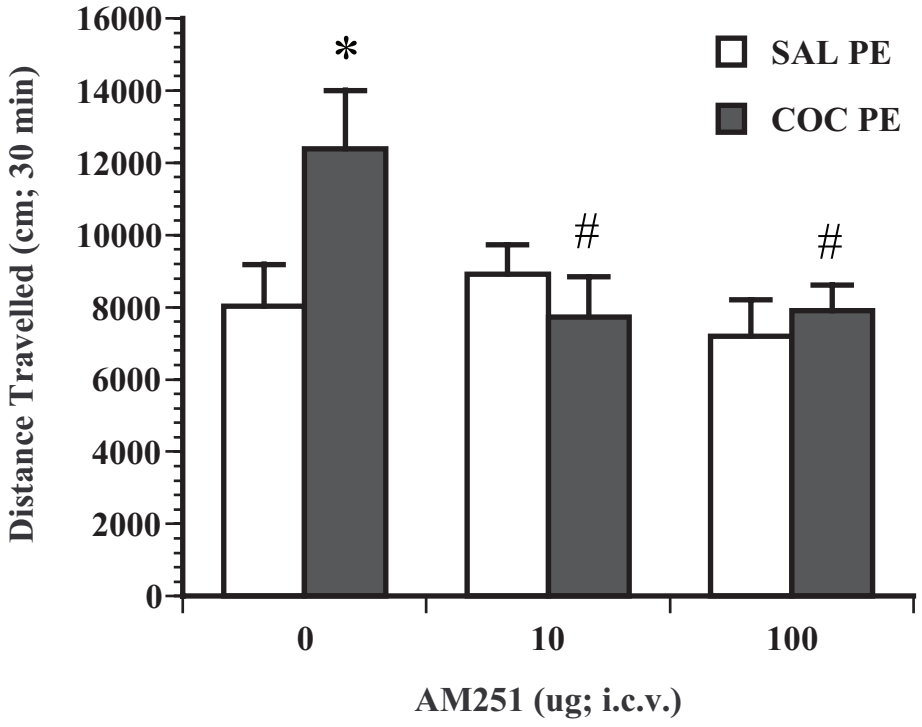
Figure 25



Mean (±SEM) distance traveled (cm) in response to a CRF challenge (0.5 µg; i.c.v.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 µg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p) [Experiment 6]. Two-way ANOVA: COC PE x AM251 PT interaction [$F(2,48)=3.36$, $p<.05$]; LSD post-hoc.

* Different from saline pre-exposure (SAL PE) condition, $p<.05$; # Different from 0 µg AM251 condition, $p<.05$.

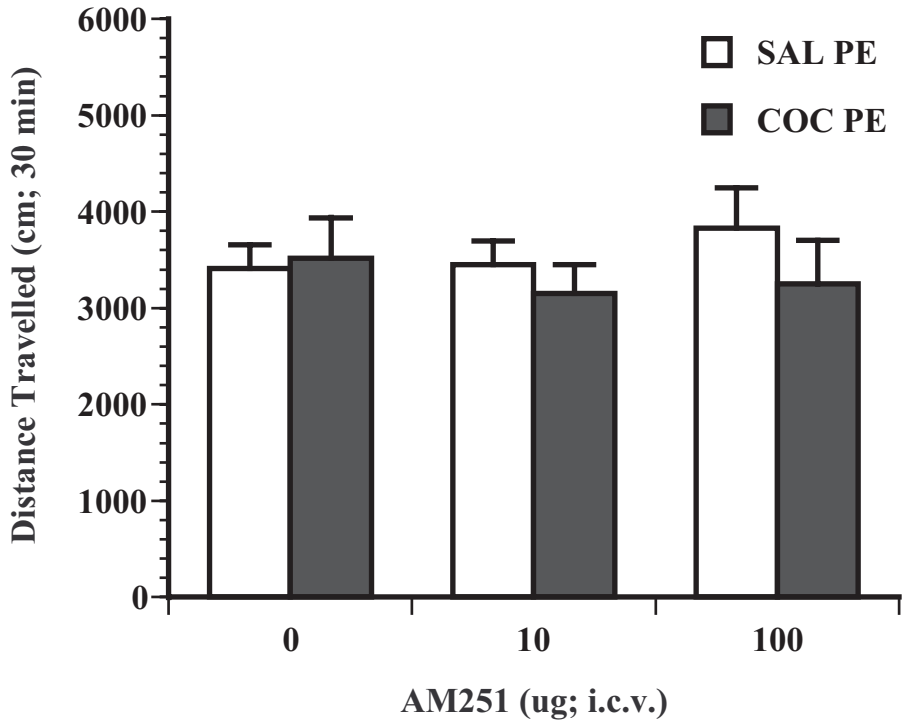
Figure 26



Mean (±SEM) distance traveled (cm) in response to a cocaine challenge (15 mg/kg; i.p.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 µg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) [Experiment 6]. Two-way ANOVA: COC PE x AM251 PT interaction [$F(2,49)=3.27$, $p<.05$]; LSD post-hoc.

* Different from saline pre-exposure (SAL PE) condition, $p<.05$; # Different from 0 µg AM251 condition, $p<.05$.

Figure 27



Mean (±SEM) distance traveled (cm) in response to a saline challenge (i.c.v.), administered 30 min after acute AM251 pretreatment (0, 10, or 100 µg; i.c.v.) and 12 days after the last cocaine pre-exposure (0 or 15-30 mg/kg; i.p.) [Experiment 6].

AM251 on footshock-induced reinstatement was not surprising based on earlier work of De Vries and colleagues (2001) demonstrating that systemic administration of the CB_1 receptor antagonist, SR141716, had no effect on footshock-induced reinstatement of cocaine seeking. Perhaps more surprising was the striking effect of AM251 on CRF-induced reinstatement of cocaine seeking - at even very low doses - and, moreover, the striking contrast in the effects of AM251 on CRF- and footshock-induced reinstatement of cocaine seeking, the latter known to be mediated by CRF (Erb et al., 1998; 2001; Erb and Stewart, 1999; Wang et al., 2005a). The dissociation between the effects of these two stressors on reinstatement is reminiscent of the results reported in Chapter 3, in which central administration of TCAP-1 completely blocked CRF-induced reinstatement of cocaine seeking over a wide dose range while having no effect on footshock-induced reinstatement. As discussed at some length in Chapter 3, several factors likely contributed to the differential regulation of CRF- and footshock-induced reinstatement, including the nature of the stressors (pharmacological vs. physical/environmental), the brain systems they engage (Imaki et al., 1993; Dunn et al., 2000), and the onset and duration of their neurochemical effects (Matsuzaki et al., 1989; Kalivas and Duffy, 1995; Galvez et al., 1996; Erb et al., 2000; de Groote et al., 2005). Thus, as discussed in the context of TCAP-1, signaling at CB_1 receptors may differentially influence transmission within pathways mediating footshock and CRF-induced reinstatement of cocaine seeking.

In the present experiments, AM251 was also found to be without effect on the reinstatement of cocaine seeking induced by a cocaine prime. In this case, however, similar null effects of CB_1 receptor antagonism on cocaine-induced reinstatement have been reported. For example, Ward and colleagues (2009) found in mice that systemic pretreatment with the CB_1 receptor antagonist, SR141716, had no effect on cocaine-induced reinstatement of cocaine seeking. Furthermore, cocaine-induced reinstatement of extinguished cocaine CPP was unaltered by systemic pretreatment with AM251 (Vaughn et al., 2011). Considerable evidence

does, however, point to a mediating role for CB_1 receptor transmission in cocaine-induced reinstatement of cocaine seeking (De Vries et al., 2001; Filip et al., 2006a). Most notably, Xi and colleagues (2006) reported that systemic and intra-striatal administration of AM251 blocked and attenuated, respectively, cocaine-induced reinstatement of cocaine seeking. The reasons for the discrepancies between these and the present findings are unclear. One possibility is that the discrepancies owe to differences in the route of AM251 administration (i.c.v. in the present experiment vs. intrastriatal or systemic in previous studies). From this perspective, it is possible that differences in dose and regional distribution of the antagonist led to different outcomes.

4.5.2. AM251 Blocked Cocaine-induced Behavioural Sensitization

In Experiment 6, AM251 was found to block the cocaine-sensitized locomotor response to both a CRF and a cocaine challenge, without itself producing any change in locomotion. These results parallel the effects of AM251 on CRF-induced reinstatement of cocaine seeking in Experiment 4, and are consistent with previous reports demonstrating a mediating role for CB_1 receptors in cocaine-induced expression of behavioural sensitization (Filip et al., 2006a; Ramiro-Fuentes and Fernandez-Espejo, 2011). Previous reports have also identified a critical role for CRF signaling in the sensitized locomotor response to cocaine, as evidenced by a blockade of the response by acute pretreatment with CRF receptor antagonists (Przegaliński et al., 2005; Erb and Brown, 2006). Taken together, this evidence suggests that CB_1 receptors play a role in mediating the expression of behavioural sensitization perhaps, in part, through their interaction with CRF systems. Possible mechanisms of an interaction between eCBs and CRF will be elaborated upon in the next sections.

4.5.3. Mechanisms of Interaction between eCBs and CRF

4.5.3.1. Potential Cellular Interactions between eCBs and CRF

The mechanisms by which eCBs and CRF interact at the cellular level are unclear. It is well established that eCB signaling at CB_1 receptors results in the suppressed release of a variety of neurochemicals, predominantly glutamate and GABA (Szabo and Schlicker, 2005). No work to date, however, has demonstrated that CB_1 receptor activation suppresses CRF release. Moreover, the potent interference by AM251 in the effects of exogenously administered CRF suggests that AM251 acts downstream of CRF receptor binding to exert its behavioural effects.

Given that CB_1 and CRF receptors are G protein-coupled, and influence many common intracellular systems, it is possible that the intracellular actions of eCBs and CRF directly interact. Indeed, CRF_1 and CRF_2 receptors act primarily through G_s proteins to activate adenylate cyclase and increase cAMP production (Grammatopoulos et al., 2001), whereas CB_1 receptors are $G_{i/o}$ protein-coupled, and thus act to inhibit adenylate cyclase and cAMP production (Freund et al., 2003). However, the opposing intracellular effects of CB_1 and CRF receptor transmission fail to account for the present findings that CB_1 receptor blockade, not activation, interferes with the effects of CRF. Moreover, CRF receptors are predominantly expressed on postsynaptic neuronal elements (Treweek et al., 2009; Jaferi and Pickel, 2009; Reyes et al., 2007), whereas CB_1 receptors are predominantly presynaptic (Freund et al., 2003); as such, a direct intracellular interaction between eCBs and CRF is unlikely.

A more plausible cellular mechanism of eCB-CRF interaction is one in which CB_1 receptor transmission indirectly modulates CRF action by altering the balance of excitatory and inhibitory tone on CRF receptor expressing neurons. As mentioned, eCBs, through their action at CB_1 receptors, are critical regulators of glutamate and GABA release (Freund et al., 2003). CRF, on the other hand, acts primarily as a neuromodulator, whereby it alters the excitability of

the postsynaptic membrane so as to enhance or suppress the effects of neurotransmitters like glutamate and GABA (Aldenhoff et al., 1983; Bishop and King, 1992; Haug and Storm, 2000; Gallagher et al., 2008; Wanat et al., 2008; Giesbrecht et al., 2010). For example, CRF, acting through CRF_1 receptors, has been shown to increase the intrinsic excitability of BLA projection neurons (Giesbrecht et al., 2010) and VTA DA neurons (Wanat et al., 2008), and potentiate their response to excitatory input (Ugolini et al., 2008; Hahn et al., 2009). As such, the cellular actions of CRF may be uniquely sensitive to eCB regulation of the balance between excitatory and inhibitory transmission.

4.5.3.2. Potential Brain Loci where AM251 Acts to Block CRF-induced Reinstatement

The brain loci in which eCBs and CRF interact to mediate reinstatement behaviour are currently unknown. Two regions that warrant consideration are the BNST and VTA, given that local infusions of CRF into these regions induce reinstatement of cocaine seeking (Erb and Stewart, 1999; Erb et al., 2001; Wang et al., 2005a). Neurons in both the BNST and VTA also express CB_1 receptors (Herkenham et al., 1991; Matyas et al., 2008) that regulate the local release of glutamate and GABA (Puente et al., 2010; Melis et al., 2004; Szabo et al., 2002; Riegel and Lupica, 2004). Thus, it is possible that AM251 modulates excitatory and inhibitory transmission in one or more of these regions to functionally block the effects of CRF on reinstatement behaviour.

Potentially problematic for the view that AM251 acts within the BNST and VTA to counter CRF-induced reinstatement is the fact that CRF signaling in both regions has been implicated in footshock-induced reinstatement, which is unaltered by CB_1 receptor antagonism (Experiment 4; De Vries et al., 2001). From this perspective, it is of interest that there are brain regions that are not implicated in footshock-induced reinstatement, but that are critical for other forms of reinstatement, express CRF and CB_1 receptors, and mediate various behaviours

induced by central CRF and CB_1 receptor transmission; such regions could help to account for the unique regulation of CRF-induced reinstatement of cocaine seeking by AM251.

One such region may be the BLA. Indeed, the BLA has been shown to densely express CRF_1 receptors (Chalmers et al., 1995; Bittencourt and Sawchenko, 2000), and its CB_1 receptor expression is considerably more pronounced than that of the BNST or VTA (Herkenham et al., 1991; Marsicano and Lutz, 1999). Moreover, pharmacological inhibition of the BLA has been shown to be without effect on footshock-induced reinstatement (McFarland et al., 2004). On the other hand, activation of the BLA by electrical stimulation and local NMDA infusion induces the reinstatement of cocaine seeking (Hayes et al., 2003). The BLA also plays a critical role in the reinstatement of cocaine seeking induced by exposure to previously cocaine-associated cues (Meil and See, 1997; Kruzich and See, 2001), an effect that is, interestingly, attenuated by both CB_1 (De Vries et al., 2001; Filip et al., 2006a; Ward et al., 2009) and CRF receptor antagonists (Goeders and Clampitt, 2002; Moffett and Goeders, 2007; Smith and Aston-Jones, 2011).

Additional support for the BLA as a likely regional substrate for the effects of AM251 on CRF-induced reinstatement comes from evidence that excitatory BLA projection neurons, known to innervate many areas implicated in reinstatement behaviours (e.g., CeA, BNST, NAc, and PFC (Dong et al., 2001; Sah et al., 2003; Knapska et al., 2007)), are under regulatory control by both CRF and eCBs. For example, activation of CRF_1 receptors on BLA projection neurons has been shown to potentiate the response of these cells to excitatory input (Ugolini et al., 2008), suppress their response to inhibitory input (Rainnie et al., 2004), and increase their intrinsic excitability (Giesbrecht et al., 2010). BLA projection neurons also receive inhibitory, perisomatic input from CB_1 receptor-expressing GABAergic terminals (Katona et al., 2001; McDonald and Mascagni, 2001; Azad et al., 2004). Accordingly, eCB signaling in the BLA suppresses GABA release onto BLA projection neurons, thereby increasing their excitability and excitatory output to terminal regions implicated in reinstatement (Katona et al., 2001; Azad

et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011). In contrast, AM251 may act within the BLA to block the eCB-mediated disinhibition of BLA neurons, thereby suppressing their excitatory output. Taken together, these findings suggest that i.c.v. CRF may act within the BLA to activate its projection neurons and induce reinstatement, and that AM251 may counter this effect by constraining the activity of these same neurons.

4.5.3.3. Potential Brain Loci where AM251 Acts to Block Sensitization

eCB regulation of CRF- and cocaine-induced expression of sensitization may be localized to a number of regions in the limbic forebrain. For example, one report showed that local administration of the CB_1 receptor antagonist, SR141716, into the NAc blocked the expression of sensitization to a cocaine challenge (Ramiro-Fuentes and Fernandez-Espejo, 2011). AM251 may similarly act within the NAc to interfere with CRF-induced expression of sensitization, given that the NAc is widely considered a critical brain substrate for behavioural sensitization (Steketee and Kalivas, 2011). In addition, however, AM251 may act within the amygdala to block the effects of CRF and cocaine on sensitization. Indeed, considerable evidence suggests that the responsivity of amygdalar neurotransmission to both CRF and cocaine is sensitized by prior cocaine experience. For example, repeated cocaine pre-exposure has been shown to potentiate CRF-induced expression of c-fos mRNA (Erb et al., 2005), and cocaine-induced release of CRF, within the CeA (Richter et al., 1995). AM251, however, is unlikely to act directly within the CeA to influence cocaine-sensitized responses to CRF and cocaine, given that CB_1 receptor expression in this region is barely detectable (Tsou et al., 1998; Katona et al., 2001). Instead, AM251 may act within the BLA, a region rich with CB_1 receptors (Marsciano and Lutz, 1999), to block the eCB-mediated disinhibition of BLA neurons projecting to the CeA (Katona et al., 2001; Azad et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011). The resulting reduction in CeA activity may dampen its sensitized response to

CRF and cocaine, thereby inhibiting the expression of sensitization. Consistent with this view, interference in normal excitatory transmission within the BLA by local infusion of the NMDA receptor antagonist, MK-801, blocked the expression of locomotor sensitization to a cocaine challenge (Kalivas and Alesdatter, 1993).

4.5.3.4. Potential Role for Dopamine in the Effects of AM251 on CRF-induced Reinstatement

Recent work from our laboratory has demonstrated that the effects of i.c.v. CRF on reinstatement are mediated, at least in part, by DA transmission. Specifically, systemic pretreatment with the D_1-like receptor antagonist, SCH23390, blocked CRF-induced reinstatement of cocaine seeking (Brown et al., unpublished). These findings are consistent with evidence of a role for DA in reinstatement more generally. Indeed, as previously mentioned, local administrations of SCH23390 and the mixed D_1/D_2-like receptor antagonist, flupenthixol, into various subregions of the PFC have been shown to block both footshock- and cocaine-induced reinstatement of cocaine seeking (McFarland and Kalivas, 2001; Capriles et al., 2003; McFarland et al., 2004). Furthermore, inactivation of the VTA by the combined, local infusion of $GABA_A$ and $GABA_B$ receptor agonists blocks both footshock- (McFarland et al., 2004; Wang et al., 2005a) and cocaine-induced reinstatement (McFarland and Kalivas, 2001).

Given the important role for DA transmission in reinstatement, it has been argued that CB_1 receptor antagonists like AM251 may modulate reinstatement behaviour by constraining VTA DA neuron activity (Lupica and Riegel, 2005). Upon activation, VTA DA neurons release eCBs that act on presynaptic CB_1 receptors to regulate glutamatergic and GABAergic input (Riegel and Lupica, 2004; Melis et al., 2004; Pan et al., 2008). eCB signaling appears to produce a net reduction in the inhibitory tone over DA neuron activity, given that CB_1 receptor agonists and eCB transport inhibitors reliably increase DA cell firing and release (Chen et al.,

1990; 1991; Gessa et al., 1998; Pillolla et al., 2007). Accordingly, CB_1 receptor antagonists reverse the increased cell firing that results from CB_1 receptor transmission (French, 1997; Gessa et al., 1998; Cheer et al., 2003), and, in so doing, may interfere with reinstatement behaviour.

Evidence further suggests that AM251 may interfere with CRF-induced reinstatement in a comparable manner. First, CRF has been shown to act within the VTA to induce the reinstatement of cocaine seeking (Wang et al., 2005a; 2007). CRF treatment has been also shown to potentiate excitatory transmission onto VTA DA neurons (Ungless et al., 2003; Hahn et al., 2009), increase their excitability and firing rate (Wanat et al., 2008), and thus promote DA signaling in various terminal regions, including the PFC, amygdala, and NAc (Lavicky and Dunn, 1993; Matsuzaki et al., 1998; Dunn, 2000). As such, it is possible that AM251, by blocking the eCB-mediated suppression of GABA release onto VTA DA neurons (Riegel and Lupica, 2004; Pan et al., 2008; Thiemann et al., 2008), may counteract the excitatory effects of i.c.v. CRF on DA neuron activity and release, and thus interfere with CRF-induced reinstatement. However, given the ubiquitous role for DA transmission in the reinstatement of drug seeking, and the importance of VTA CRF for reinstatement induced by footshock stress (Wang et al., 2005a), it is unclear how AM251 acting within the VTA could account for the present selectivity of AM251 to interfere with CRF-induced reinstatement.

4.5.3.5. Potential Role for Dopamine in the Effects of AM251 on Sensitization

Perhaps more plausible is the notion that AM251 interferes with the expression of sensitization to challenge injections of cocaine and CRF via its effects on DA transmission. Psychostimulant-induced expression of behavioural sensitization has long been associated with the enhanced release of DA in the VTA, NAc, and amygdala (Harmer et al., 1997; Vanderschuren and Kalivas, 2000; Steketee and Kalivas, 2011). Contributing to this enhanced

release is an increase in the excitability of VTA DA neurons that develops with repeated psychostimulant exposure (White and Wang, 1984; Henry et al., 1989). Similarly, the capacity for CRF to increase the excitability of DA neurons (Ungless et al., 2003; Wanat et al., 2008) and promote DA release (Matsuzaki et al., 1989; Lavicky and Dunn, 1993) has been shown to sensitize with repeated cocaine exposure (Hahn et al., 2009; Wang et al., 2005a). As such, it is possible that AM251, by blocking the eCB-mediated suppression of GABA release onto VTA DA neurons (Riegel and Lupica, 2004; Pan et al., 2008; Thiemann et al., 2008), may reduce the heightened excitability of these neurons in cocaine pre-exposed animals, and thereby interfere with the expression of behavioural sensitization induced by either cocaine or CRF.

4.5.3.6. Potential Non-specific Effects of AM251 on Reinstatement or Sensitization

Although considered a selective CB_1 receptor antagonist, AM251 has been shown to exhibit a more complex pharmacological profile. Indeed, AM251, like many CB_1 receptor antagonists, behaves under some conditions as an inverse agonist at CB_1 receptors (Pertwee, 2005). Inverse agonism is unlikely to account for the present effects of AM251. As explained by De Vries and colleagues (2001), inverse agonistic effects of CB_1 receptor agents involve the inhibition of constitutively active CB_1 receptors, and should therefore be most pronounced under basal conditions, when activity-dependent mobilization of eCBs is low. In the present studies, however, AM251 had no behavioural effects when administered alone (i.e. saline, no footshock conditions), but had inhibitory effects only when paired with an activating agent (i.e. cocaine, CRF conditions). In addition, AM251 has been shown to act as an antagonist at adenosine A_1 receptors (Savinainen et al., 2003). Any blockade of adenosine signaling, however, is unlikely to have contributed to the present effects of AM251 on reinstatement or sensitization, given that adenosine receptor antagonists have in fact been shown to reinstate

cocaine seeking behaviour (Weerts and Griffiths, 2003) and enhance cocaine-induced expression of sensitization (Filip et al., 2006b).

CHAPTER 5

Role of Endocannabinoid CB_1 Receptors in CRF- and Cocaine Withdrawal-induced Anxiety

CHAPTER 5

Role of Endocannabinoid CB_1 Receptors in CRF- and Cocaine Withdrawal-induced Anxiety

5.1. Introduction

CRF plays an important role in the central regulation of behavioural anxiety, and appears to do so primarily via its actions at extrahypothalamic brain sites (see Sawchenko et al., 1993; Sarnyai et al., 2001; Rodaros et al., 2007). For example, i.c.v. injections of CRF induce anxiety in the EPM and other behavioural tests (e.g. Baldwin et al., 1991; Spina et al., 2002), and these responses are unaltered by manipulations of HPA activity, such as hypophysectomy, dexamethasone pretreatment, or PVN lesions (Eaves et al., 1985; Britton et al., 1986; Berridge and Dunn, 1989; Liang et al., 1992; Pich et al., 1993). Similarly, acute (24-48h) withdrawal from several drugs of abuse induces anxiety that is mediated by amygdalar CRF transmission (Rassnick et al., 1993; Sarnyai et al., 1995; Zhou et al., 1996; DeVries and Pert, 1998; Richter and Weiss, 1999; Zorrilla et al., 2001; Erb et al., 2003; see Section 1.2.3).

eCBs, such as AEA and 2-AG, also play a critical role in regulating behavioural anxiety, primarily via their actions at presynaptic CB_1 receptors. In general, pharmacological enhancement of CB_1 receptor transmission is anxiolytic (e.g. Patel and Hillard, 2006; Rubino et al., 2007; but see Griebel et al., 2005), whereas pharmacological suppression of CB_1 transmission is anxiogenic (e.g. Haller et al., 2004; Rodgers et al., 2005; but see Lafenêtre et al., 2007); however, as described in Chapter 1, these behavioural effects are brain region-specific (Onaivi et al., 1995; Moreira et al., 2007; Roohbakhsh et al., 2007; Rubino et al., 2008a; b; Zarrindast et al., 2008). In addition, evidence points to a bidirectional, region-specific role for eCB signaling in the negative regulation of the HPA axis; specifically, downregulation of tonic AEA signaling in the BLA in response to stress promotes HPA activity (Hill et al., 2009), whereas stress-induced upregulation of 2-AG signaling in the hypothalamus (Evanson et al.,

2010) and PFC (Hill et al., 2011) seems to suppress HPA activity (see Chapter 1, Hill and McEwen, 2010 for review).

The eCB system has also been shown to regulate behavioural anxiety related to prior drug and ethanol administration. For example, in a manner that mimicked the effects of CRF receptor antagonists (Baldwin et al., 1991; DeVries and Pert, 1998), administration of the CB_1 receptor antagonist, SR141716, before testing in the EPM reversed the reduction in time spent in the open arms by rats withdrawn from chronic exposure to ethanol (Rubio et al., 2008; Onaivi, 2008), diazepam, or cocaine (Onaivi, 2008).

Given the key roles that CRF and eCBs play in regulating anxiety, it is plausible that these systems interact to mediate anxiety-like responses. Consistent with this idea, *in situ* hybridization studies reveal a high degree of colocalization between the mRNA of CB_1 receptors and both CRF and CRF_1 receptors within cortical and limbic brain regions known to be important for stress regulation (Hermann and Lutz, 2005; Cota et al., 2007). In fact, hypothalamic CRF neurons have been shown to be under the regulatory control of eCB signaling (Di et al., 2003; Evanson et al., 2010). Experiments in Chapter 4 also revealed that CB_1 receptor transmission is critical for the expression of several behaviours induced or mediated by CRF. Moreover, this functional interaction between eCB and CRF signaling has been proposed to occur at the level of the BLA, a brain region commonly implicated in the regulation of anxiety-like behaviour.

Accordingly, the experiments presented in this chapter were designed to explore the effects of the CB_1 receptor antagonist, AM251, on behavioral anxiety induced by i.c.v. injections of CRF (Experiment 7A), and withdrawal from chronic exposure to cocaine (mediated by CRF; Experiment 8A). In Experiments 7B and 8B, plasma corticosterone levels were assessed to explore whether any behavioural effects observed in the corresponding studies were related to changes in HPA axis activity. The present findings show that AM251 reversed

the behavioural anxiety induced by CRF and withdrawal from chronic cocaine administration in a manner that did not parallel its effects on plasma corticosterone. These results suggest that the anxiogenic effects of CRF and cocaine withdrawal are mediated by CB_1 receptor transmission, independent of HPA axis regulation.

5.2. Experiment 7A: Effects of AM251 on CRF-induced Anxiety

5.2.1. Materials and Methods

5.2.1.1. Subjects

A total of 108 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

5.2.1.2. Surgery

Rats were surgically implanted with an i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2.1).

5.2.1.3. Apparatus

Anxiety testing was carried out in the elevated plus maze described in Chapter 2 (Section 2.5.4).

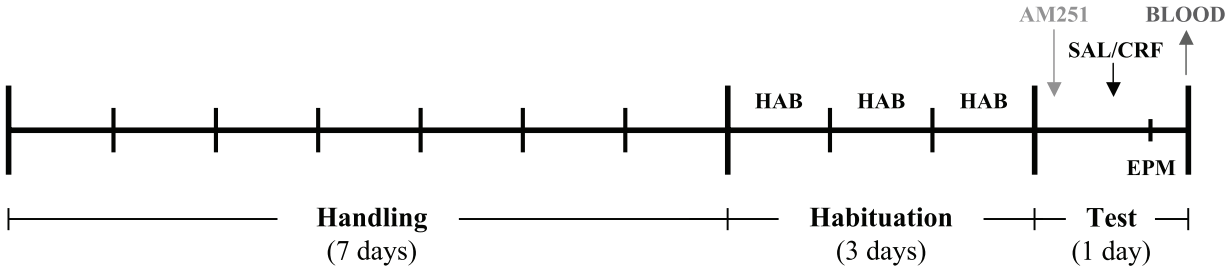
5.2.1.4. Drugs

CRF (Sigma-Aldrich) was dissolved in sterile, physiological saline. AM251 (Tocris Bioscience) was dissolved in DMSO (Sigma-Aldrich). The dose range and vehicle used for AM251 was based on the results of Experiments 4, 6A, and 6B, as well as previous work (Sink et al., 2009). Central injections of CRF and AM251 were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

5.2.1.5. Procedures

Figure 28 shows the procedural timeline of Experiments 7A and 7B.

Figure 28



Procedural timeline for Experiments 7A and 7B.

5.2.1.5.1. Habituation

On each of three days prior to testing in the EPM, rats were transported in their home cages to a darkened room adjacent the testing room, where they were left undisturbed for 30 min. Rats were then given two sham i.c.v. injections separated by 30 min and, after an additional 30-min period, were returned to the colony room.

5.2.1.5.2. Test for Anxiety

On the test day, rats were again transported to the darkened room and left undisturbed for 30 min. They were then injected with AM251 (0, 10, 100, or 200 μg, i.c.v.) and, 30 min later, with CRF (0 or 0.5 μg, i.c.v.). After an additional 30 min, rats were placed individually onto the center platform of the EPM, facing towards a closed arm, and allowed to explore the maze freely for 5 min. Video footage of the testing was captured, and an observer blind to the treatments later scored the footage for entries made into, and time spent in the open and closed arms.

5.2.2. Results

Figure 29 shows the mean (±SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 μg; i.c.v.) and CRF (0 [SAL] or 0.5 μg; i.c.v.). Two-way ANOVA of time spent in the open arms revealed a significant interaction of AM251 x CRF [$F(3,108)=5.70$, $p<.001$]. CRF treatment reduced open arm time in rats pretreated with 0 μg, but not 10, 100, or 200 μg AM251 ($p<.001$). Rats pretreated with 100 or 200 μg AM251 prior to a CRF challenge spent significantly more time in the open arms than those pretreated with 0 μg AM251 ($p<.01$). In addition, at the highest dose (200 μg), AM251 reduced time spent in the open arms ($p<.05$). Analyses of the percent ratio of entries into the open relative to closed arms revealed similar results, including a significant AM251 x CRF interaction [$F(3,106)=4.21$,

p<.01], and comparable significant post-hoc comparisons (p<.05). Collectively, these results indicate that AM251, while anxiogenic at the highest dose, reversed the anxiety-like behaviour induced by i.c.v. CRF.

Table 1 shows the mean (±SEM) number of open and closed arm entries made during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 µg; i.c.v.) and CRF (0 or 0.5 µg; i.c.v.). Two-way ANOVA of open arm entries revealed a significant AM251 x CRF interaction [$F(3,108)=5.06$, p<.005]. Comparable to the dependent measures depicted in Figure 29, open arm entries were significantly reduced by CRF in rats pretreated with 0 µg AM251 (p<.001), and by the highest dose of AM251 in saline treated rats (p<.05). Furthermore, pretreatment with 100 or 200 µg AM251 blocked CRF-induced reductions in open arm entries (p's<.05). In contrast, two-way ANOVA of closed and total arm entries revealed only overall reductions in rats given CRF [closed: $F(1,106)=7.11$, p<.01; total: $F(1,106)=9.47$, p<.005].

5.3. Experiment 7B: Effects of AM251 on CRF-induced Plasma Corticosterone

5.3.1. Materials and Methods

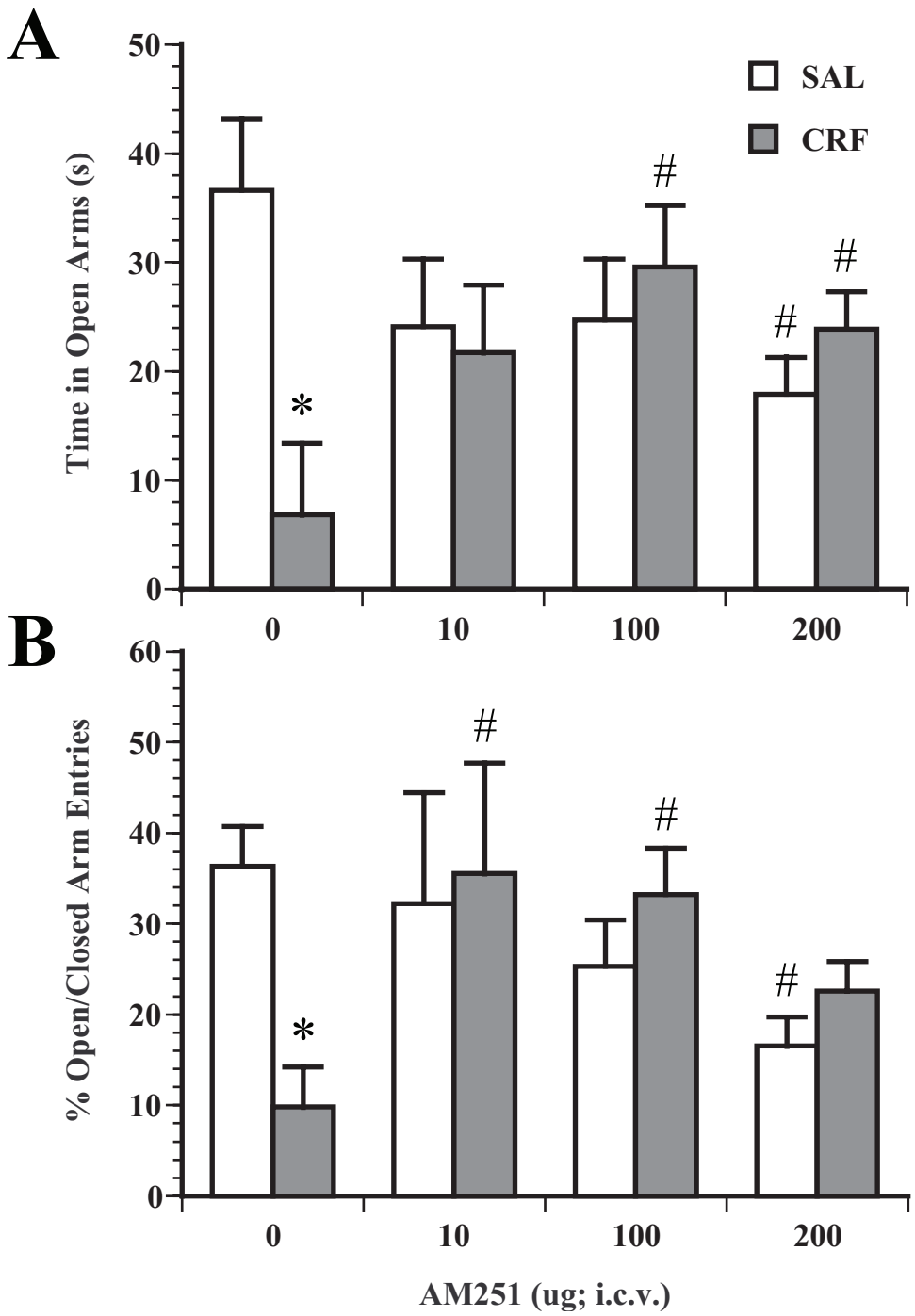
5.3.1.1. Subjects

A total of 63 rats from Experiment 7A in this chapter were used in this study.

5.3.1.2. Procedures

Twenty-five minutes following testing in the EPM, rats were anaesthetized with isoflurane, and sacrificed by decapitation. Trunk blood (2 ml) was collected and chilled to 4°C on ice. Samples were then centrifuged (6000 rpm; 4°C) for 5 minutes and plasma was stored at -80°C. Immediately following decapitation, brains were extracted, frozen in -40°C isopentane, and stored at -80°C. Accurate cannula placements were subsequently confirmed by histological analysis.

Figure 29



Mean (±SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 µg; i.c.v.) and CRF (0 [SAL] or 0.5 µg; i.c.v.) [Experiment 7A]. (A) Two-way ANOVA: AM251 x CRF interaction [$F(3,108)=5.70$, $p<.001$]; LSD post-hoc. (B) Two-way ANOVA: AM251 x CRF interaction [$F(3,106)=4.21$, $p<.01$]; LSD post-hoc.

* Different from SAL condition, $p<.05$; # Different from 0 µg AM251 condition, $p<.05$.

Table 1

	Open Arm Entries				Closed Arm Entries			
Treatment	0	10	100	200	0	10	100	200
SAL	4.0 (0.5)	2.7 (0.6)	2.9 (0.6)	1.7 (0.3) #	11.1 (0.8)	10.3 (1.1)	11.6 (0.7)	11.9 (0.8)
CRF	0.9 (0.3) *	2.4 (0.8)	2.7 (0.4) #	2.5 (0.7) #	9.9 (0.7)	9.1 (1.5)	8.6 (0.4)	10.9 (0.7)

Mean (±SEM) number of open and closed arm entries made during a 5-min test in the EPM by rats following administration of AM251 (0, 10, 100, or 200 µg; i.c.v.) and CRF (0 or 0.5 µg; i.c.v.) [Experiment 7A]. Two-way ANOVA: AM251 x CRF interaction [$F(3,108)=5.06$, $p<.005$]; LSD post-hoc.

* Different from SAL condition, $p<.05$; # Different from 0 µg AM251 condition, $p<.05$.

Plasma corticosterone was measured using a double-antibody ^{125}I Radioimmunoassay (ImmuChem 07-120103, MP Biomedicals), which has been used extensively for rat plasma (e.g. Sithisarn et al., 2011). Manufacturer's directions were followed except that the volumes of all reagents were halved and plasma diluted 1:300 (0.16 μL of plasma per tube). Each sample was measured in duplicate.

5.3.2. Results

Figure 30 shows the mean (±SEM) plasma corticosterone levels in rats following administration of AM251 (0, 10, 100, or 200 μg; i.c.v.) and CRF (0 or 0.5 μg; i.c.v.), and EPM testing. Two-way ANOVA of plasma corticosterone levels revealed a significant main effect of AM251 [$F(3,59)=3.07$, $p<.05$], attributable to elevated levels at all doses of AM251 relative to the 0 μg condition (p's<.05). Pearson correlations between performance on each EPM measure (Experiment 7A) and plasma corticosterone levels were all non-significant.

5.4. Experiment 8A: Effects of AM251 on Cocaine Withdrawal-induced Anxiety

5.4.1. Materials and Methods

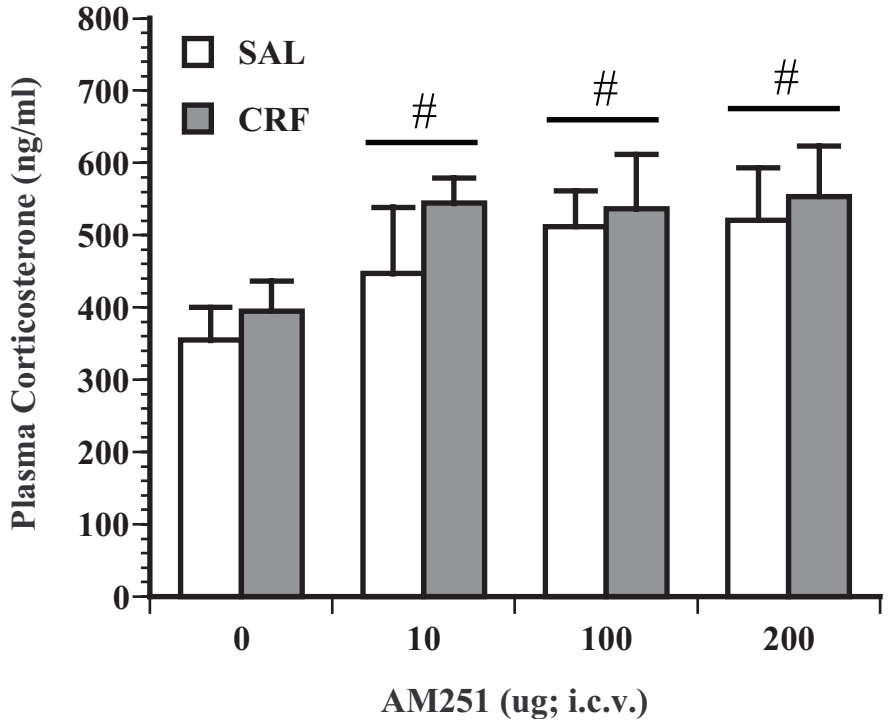
5.4.1.1. Subjects

A total of 63 male Long Evans rats (250-275g), housed and maintained under the conditions described in Chapter 2 (Section 2.1), were used in this experiment.

5.4.1.2. Surgery

Rats were surgically implanted with an i.c.v. cannula using the procedures described in Chapter 2 (Section 2.2.1).

Figure 30



Mean (±SEM) plasma corticosterone levels in rats following administration of AM251 (0, 10, 100, or 200 µg; i.c.v.) and CRF (0 or 0.5 µg; i.c.v.), and EPM testing [Experiment 7B]. Two-way ANOVA: Main effect of AM251 [$F(3,59)=3.07$, $p<.05$]; LSD post-hoc.

Different from 0 µg AM251 condition, $p<.05$.

5.4.1.3. Apparatus

Anxiety testing was carried out in the elevated plus maze described in Chapter 2 (Section 2.5.4).

5.4.1.4. Drugs

Cocaine HCl (Medisca Pharmaceuticals) and CRF (Sigma-Aldrich) were dissolved in sterile, physiological saline. AM251 (Tocris Bioscience) was dissolved in DMSO (Sigma-Aldrich). The dose range and vehicle used for AM251 was based on the results of Experiments 4 and 7A, as well as previous work (Sink et al., 2009). Central injections of CRF and AM251 were conducted according to the microinjection procedures described in Chapter 2 (Section 2.4).

5.4.1.5. Procedures

Figure 31 shows the procedural timeline of Experiments 8A and 8B.

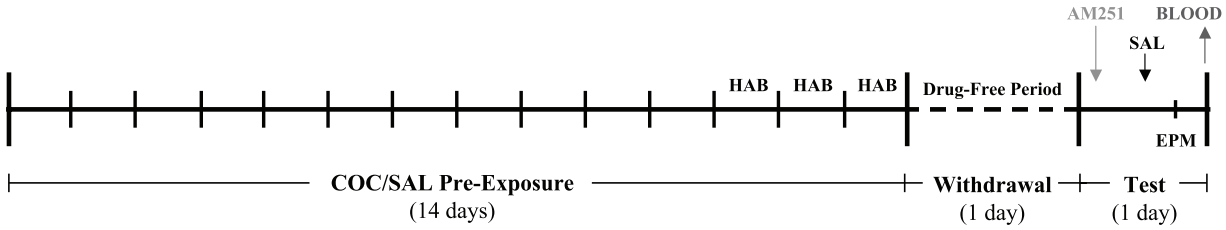
5.4.1.5.1. Cocaine Pre-Exposure

Seven days after surgery, rats were injected daily with cocaine (20 mg/kg, i.p.) or saline for 14 days. All injections were given during the dark phase of the light cycle, within 1-2 hours of the time of day that their subsequent testing was performed. Rats were handled for 2 min on each pre-exposure day to reduce their basal anxiety. On each of the three final pre-exposure days, rats were habituated to the transport and injection procedures used during anxiety testing. Specifically, rats were transported in their home cages to a darkened room adjacent the testing room, where they were left undisturbed for 30 min. Rats were then given a sham i.c.v. injection. Thirty minutes later, they were given a cocaine or saline injection and, after an additional 30 min, were returned to the colony room.

5.4.1.5.2. Test for Anxiety

Forty-eight hours after the last cocaine/saline injection, corresponding to high levels of withdrawal-induced anxiety (e.g., Sarnyai et al., 1995), rats were tested in the EPM. Rats were transported to the darkened room adjacent the testing room and left undisturbed for 30 min.

Figure 31



Procedural timeline for Experiments 8A and 8B.

Rats were then injected with AM251 (0, 10, or 100 µg, i.c.v.). Thirty minutes later, all rats were injected with saline (i.p.) to simulate the procedures used during the pre-exposure phase. After an additional 30 min, rats were given a 5-min test in the EPM, as described in Experiment 7A.

5.4.2. Results

Figure 32 shows the mean (±SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats administered AM251 (0, 10, or 100 µg; i.c.v.) 48 h following 14-day pre-exposure to saline or cocaine (20 mg/kg; i.p.). Two-way ANOVA of time spent in the open arms revealed a significant main effect of AM251 [$F(2,54)=5.04$, $p<.01$]. Rats given 10 µg AM251, relative to those given 0 or 100 µg, spent more time in the open arms (p's$<.05$). Although the AM251 x Pre-Exposure interaction was not significant, inspection of Figure 32 shows that the increased time spent in the open arms after AM251 pretreatment was apparent in cocaine, but not saline, pre-exposed rats. Two-way ANOVA of the percent ratio of entries into the open relative to closed arms revealed, in addition to a significant main effect of AM251 [$F(2,54)=5.77$, $p<.01$], a significant interaction of AM251 x Pre-Exposure [$F(2,54)=3.22$, $p<.05$]. Rats pre-exposed to cocaine and pretreated with 10 µg AM251 made proportionately more entries into the open relative to closed arms than did rats pre-exposed to cocaine and pretreated with 0 µg or 100 µg AM251 (p's$<.005$). Consistent with the effects of AM251 pretreatment in Experiment 7A, the highest dose of AM251 (in this case in saline pre-exposed rats) reduced the ratio of open to closed arm entries ($p<.05$). Collectively, these results indicate that AM251, despite having anxiogenic effects at the highest dose, reversed the anxiety-like behaviour induced by cocaine withdrawal.

Table 2 shows the mean (±SEM) number of open and closed arm entries made during a 5-min test in the EPM by saline or cocaine (20 mg/kg; i.p.) pre-exposed rats administered

AM251 (0, 10, or 100 µg; i.c.v.). Two-way ANOVA of open arm entries revealed a significant main effect of AM251 [$F(2,54)=3.89$, $p<.05$], attributable to increased entries by rats pretreated with 10 µg, relative to 100 µg AM251 ($p<.01$). In contrast, two-way ANOVA for closed and total arm entries revealed no effects.

5.5. Experiment 8B: Effects of AM251 on Cocaine Withdrawal-induced Plasma Corticosterone

5.5.1. Materials and Methods

5.5.1.1. Subjects

A total of 63 rats from Experiment 8A in this chapter were used in this study.

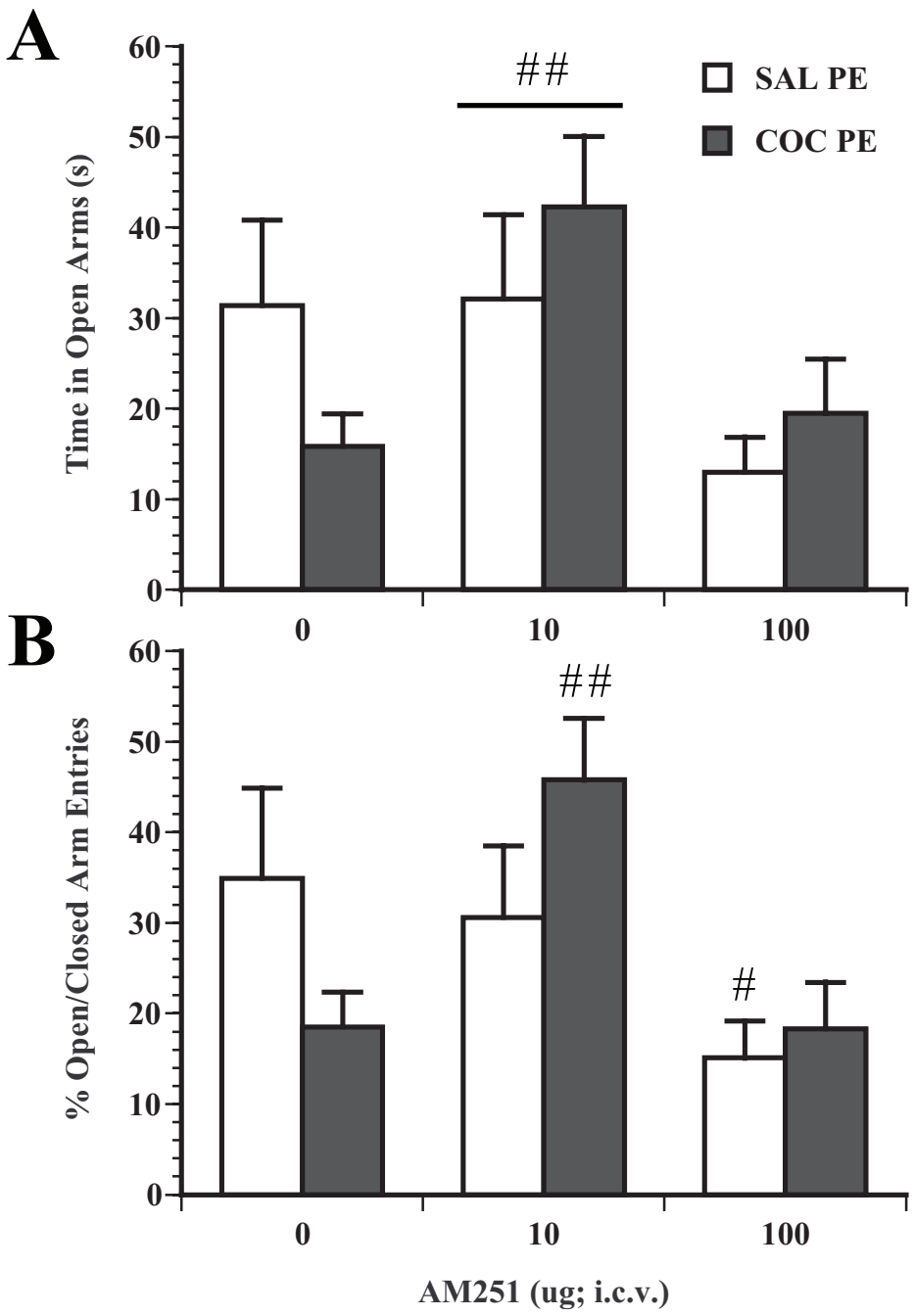
5.5.1.2. Procedures

Twenty-five minutes following testing in the EPM, trunk blood and brains were collected as described in Experiment 7B. Accurate cannula placements were subsequently confirmed by histological analysis, and plasma corticosterone levels were measures as described in Experiment 7B.

5.5.2. Results

Figure 33 shows the mean (±SEM) plasma corticosterone levels in saline and cocaine (20 mg/kg; i.p.) pre-exposed rats following AM251 (0, 10, or 100 µg; i.c.v.) administration and EPM testing. Two-way ANOVA of plasma corticosterone levels revealed a significant main effect of AM251 [$F(2,57)=3.50$, $p<.05$], attributable to elevated levels at the 10 µg relative to 0 µg dose ($p<.05$). Elevated levels of corticosterone in the group given 100 µg AM251, relative to 0 µg, approached significance ($p=.062$). Pearson correlations between performance on each EPM measure (Experiment 8A) and plasma corticosterone levels were all non-significant.

Figure 32



Mean (±SEM) time spent in the open arms (A) and percent ratio of open to closed arm entries (B) during a 5-min test in the EPM by rats administered AM251 (0, 10, or 100 µg; i.c.v.) 48 h following 14-day pre-exposure to saline or cocaine (20 mg/kg; i.p.) [Experiment 8A]. (A) Two-way ANOVA: Main effect of AM251 [$F(2,54)=5.04$, $p<.01$]; LSD post-hoc. (B) Two-way ANOVA: Main effect of AM251 [$F(2,54)=5.77$, $p<.01$], AM251 x Pre-Exposure interaction [$F(2,54)=3.22$, $p<.05$]; LSD post-hoc. # Different from 10 µg AM251 condition, $p<.05$; ## Different from 0 µg and 10 µg AM251 conditions, $p<.05$.

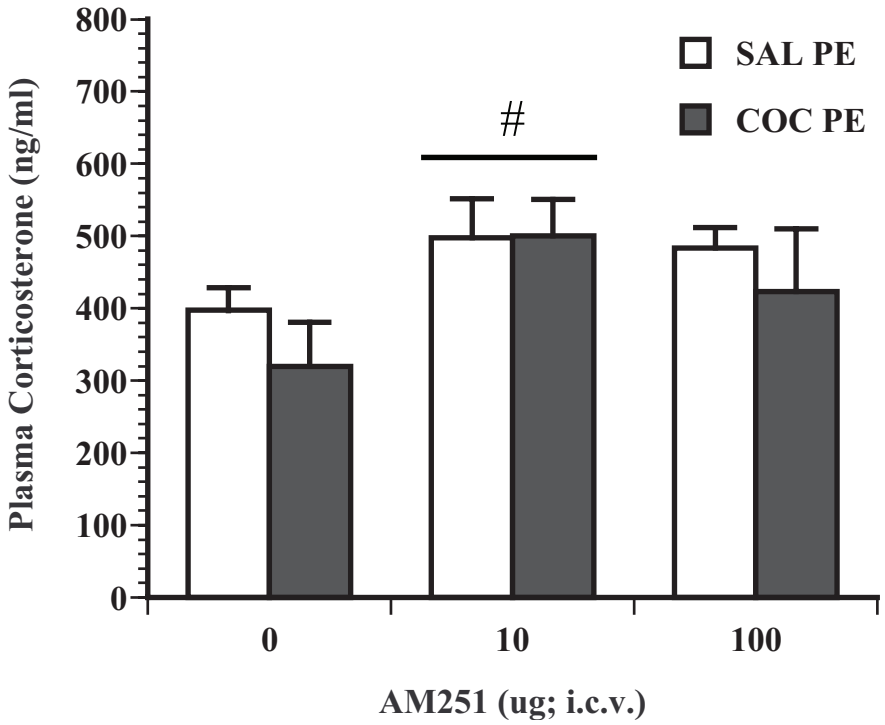
Table 2

	Open Arm Entries			Closed Arm Entries		
Treatment	0	10 #	100	0	10	100
SAL PE	3.4 (1.0)	3.6 (1.0)	1.7 (0.5)	9.9 (0.9)	11.0 (0.7)	10.4 (0.9)
COC PE	2.1 (0.5)	4.3 (0.8)	2.1 (0.6)	11.7 (0.8)	9.3 (1.1)	11.6 (0.9)

Mean (±SEM) number of open and closed arm entries made during a 5-min test in the EPM by saline or cocaine (20 mg/kg; i.p.) pre-exposed rats administered AM251 (0, 10, or 100 µg; i.c.v.) [Experiment 8A]. Open Arm Entries Two-way ANOVA: Main effect of AM251 [$F(2,54)=3.89$, $p<.05$]; LSD post-hoc.

Different from 100 µg AM251 condition, $p<.05$.

Figure 33



Mean (±SEM) plasma corticosterone levels in saline and cocaine (20 mg/kg; i.p.) pre-exposed rats following AM251 (0, 10, or 100 µg; i.c.v.) administration and EPM testing [Experiment 8B]. Two-way ANOVA: Main effect of AM251 [$F(2,57)=3.50$, $p<.05$]; LSD post-hoc.
Different from 0 µg AM251 condition, $p<.05$.

5.6. Discussion

The experiments presented in this chapter demonstrated that the CB_1 receptor antagonist, AM251, although anxiogenic in the EPM, reversed behavioral anxiety induced by CRF (Experiment 7A) and withdrawal from chronic cocaine administration (Experiment 8A) in a dose-dependent manner. Furthermore, although AM251 treatment elevated plasma corticosterone levels in both Experiments 7B and 8B, it did so irrespective of whether animals were treated with i.c.v. CRF or withdrawn from cocaine. Therefore, the effects of AM251 administration on plasma corticosterone did not parallel or correlate with its effects on CRF- or cocaine withdrawal-induced anxiety, suggesting that the anxiogenic effects of CRF and cocaine withdrawal are mediated by CB_1 receptor transmission, independent of HPA axis regulation.

5.6.1. CRF, Cocaine Withdrawal, and AM251 had Anxiogenic Effects in the EPM

Aside from a modest CRF-induced reduction in closed arm entries, it was found that CRF, cocaine withdrawal, and AM251 selectively modified open arm time and entries in the EPM. Thus, the effects of each condition are attributable to changes in anxiety-like behaviour, rather than changes in overall activity. These anxiogenic effects of i.c.v. CRF and cocaine withdrawal seen in Experiments 7A and 8A are in line with previous studies using similar dose and pre-exposure conditions (Spina et al., 2002; Sarnyai et al., 1995; Basso et al., 1999). Likewise, the anxiogenic effects of i.c.v. AM251 seen in both experiments are consistent with evidence that systemic administration of AM251 and other CB_1 receptor antagonists induce anxiety-like behavior in the EPM (Navarro et al., 1997; Rodgers et al., 2005; Sink et al., 2010).

5.6.2. AM251 Blocked the Anxiogenic Effects of CRF and Cocaine Withdrawal

The ability of AM251 to block the anxiogenic effects of CRF and cocaine withdrawal may seem at odds with a preponderance of evidence supporting an anxiolytic role for central

eCB signaling (Patel and Hillard, 2006; Hill and McEwen, 2010). However, the present effects of AM251 are in fact consistent with several recent findings pointing to a facilitatory, rather than inhibitory, role for eCB signaling in some stress-related behaviors. Indeed, in Experiment 4, AM251 was found to block the effects of CRF on the reinstatement of cocaine seeking and expression of behavioural sensitization. Previous studies have further shown that systemic pretreatment with the CB_1 receptor antagonist, SR141716, reversed behavioral anxiety in the EPM induced by withdrawal from ethanol (Rubio et al., 2008; Onaivi, 2008), diazepam, or cocaine (Onaivi, 2008). Moreover, CB_1 receptor-deficient mice with a history of ethanol self-administration failed to show any somatic signs of acute withdrawal, or an increase in ethanol consumption following exposure to footshock stress, relative to their wild-type counterparts (Racz et al., 2003). CB_1 receptor transmission has also been shown to mediate behavioural effects of stress in drug-naïve subjects. For example, pharmacological blockade of CB_1 receptors, either systemically or locally in the dorsolateral PAG or BLA, prevented the analgesic response to footshock stress (Hohmann et al., 2005; Connell et al., 2006; Kurrikoff et al., 2008).

5.6.3. Cocaine Experience Did Not Alter the Functional eCB-CRF Interaction

Exposure to cocaine has been shown to modify signaling within both the eCB and CRF systems. For example, Gonzalez and colleagues have shown that 10 days of repeated, daily cocaine injections decreased 2-AG content in the NAc and amygdala (2002a), and reduced CB_1 receptor mRNA expression in the neocortex and ventromedial hypothalamus (2002b). As described in Chapter 1, repeated cocaine exposure induces pronounced changes in amygdalar CRF content, mRNA expression, and receptor binding (Richter and Weiss, 1999; Ambrosio et al., 1997; Erb et al., 2003). Furthermore, exposure to cocaine dysregulates the eCB and CRF systems such that many subsequent cocaine-related behaviours come to be induced, or mediated, by CB_1 and CRF receptor transmission (De Vries et al., 2001; Filip et al., 2006a; Erb et al.,

1998; 2003; Erb and Brown, 2006). Despite this known dysregulation, the findings presented in this chapter suggest that the manner in which eCBs and CRF interact to mediate anxiety-like behaviour is unaffected by prior cocaine experience, given that AM251 had comparable inhibitory effects on anxiety induced by CRF in cocaine-naïve subjects, and by CRF-mediated withdrawal in cocaine-experienced subjects.

5.6.4. AM251 Increased Plasma Corticosterone Levels

In both Experiments 7B and 8B, AM251, but not CRF or cocaine withdrawal, reliably increased plasma corticosterone content. This effect of AM251 is consistent with the idea that eCB signaling negatively regulates activation of the HPA axis (Hill and McEwen, 2010). Specifically, CB_1 receptor blockade may drive HPA activity by mimicking stress-induced reductions in AEA content (Hill et al., 2009), and prevent 2-AG signaling that would otherwise serve to terminate HPA activity (Di et al., 2003; Evanson et al., 2010; Hill and McEwen, 2010). Although i.c.v. CRF (Dunn and File, 1987; Campbell et al., 2004) and acute cocaine withdrawal (Zhou et al., 2003a, 2010) have been previously found to increase plasma corticosterone, the present lack of any such increase may be attributed to the fact that blood was collected from rats after testing in the EPM. Indeed, exposure to the EPM has, itself, been shown to increase plasma corticosterone levels (Suchecki et al., 2002). Therefore, it is possible that the EPM test sufficiently engaged the HPA axis so as to render any CRF- or cocaine withdrawal-induced corticosterone release undetectable.

5.6.5. Effects of AM251 on Plasma Corticosterone Did Not Parallel Effects on Anxiety

As mentioned, our observed changes in HPA activity did not parallel or correlate with changes in behavioral anxiety; that is, the effects of AM251 on CRF- and cocaine withdrawal-induced anxiety were not reflected in plasma corticosterone content. These results suggest that

while engagement of the HPA axis accounts, at least in part, for the anxiogenic effects of AM251 (which did lead to elevations in plasma corticosterone), the effects of AM251 on CRF- and cocaine withdrawal-induced anxiety are independent of HPA axis regulation. This interpretation is strengthened by evidence from several studies showing that CRF and CRF-mediated stressors can induce anxiety in animals with compromised HPA signaling (Britton et al., 1986; Berridge and Dunn, 1989; Pich et al., 1993). Moreover, the AM251-induced reversal of anxiety-like behavior is likely mediated via an interaction with extrahypothalamic CRF systems, given that CRF transmission in the extended amygdala mediates anxiety-like behavior induced by CRF and drug withdrawal (Lee and Davis, 1997; Walker et al., 2009; Rassnick et al., 1993; Sarnyai et al., 1995), and that eCB and CRF systems show pronounced overlap in extrahypothalamic brain regions known to be important for stress regulation (Herkenham et al., 1991; Sawchenko et al., 1993).

5.6.6. Mechanisms of Interaction between eCBs and CRF on Anxiety

5.6.6.1. Potential Brain Loci where eCBs and CRF Interact to Mediate Anxiety

As described in Chapter 1, the BNST (Lee and Davis, 1997; Sahuque et al., 2006) and BLA (Sajdyk et al., 1999; Walker et al., 2009) have been identified as regions important for mediating i.c.v. CRF-induced anxiety, and the CeA as critical for the expression of cocaine withdrawal-induced anxiety (Sarnyai et al., 1995; Zhou et al., 1996; Zorrilla et al., 2001; Erb et al., 2003; Maj et al., 2003). In contrast, the brain sites involved in the effects of CB_1 receptor agonists and antagonists on anxiety are unclear. Overall, local administration of CB_1 receptor antagonists into specific brain nuclei (e.g. PFC, CeA, BLA) is largely without effect on anxiety (Rubino et al., 2008b; Zarrindast et al., 2008; Ganon-Elazar and Akirav, 2009), whereas local CB_1 receptor agonists induce bidirectional, region-specific anxiety effects (Roohbakhsh et al., 2007; Rubino et al., 2008a; b; Zarrindast et al., 2008).

The brain loci in which eCBs and CRF interact to alter anxiety-like behaviour are currently unknown. However, CB_1 receptor mRNA is colocalized with the mRNA of both CRF (Cota et al., 2007) and the CRF_1 receptor (Hermann and Lutz, 2005) in regions such as the PFC, BLA, CeA, BNST, and hippocampus. Thus, it is possible that AM251 modulates excitatory and inhibitory transmission in one or more of these regions (Auclair et al., 2000; Chiu et al., 2010; Katona et al., 2001; Domenici et al., 2006) to functionally block the effects of CRF and CRF-dependent cocaine withdrawal on anxiety-like behavior. One plausible scenario is that AM251 acts at CB_1 receptors in the dorsal hippocampus and/or BLA, regions where CB_1 receptor transmission induces behavioral anxiety (Roohbakhsh et al., 2007; Rubino et al., 2008a), to counter the anxiogenic effects of CRF and cocaine withdrawal.

In fact, the BLA represents a particularly likely regional substrate for the present effects of AM251, given its unique regulation of behavioural and neuroendocrine stress responses. Indeed, CB_1 receptor transmission in the BLA has been shown to have opposing effects on behavioural anxiety and HPA axis activity, similar to the effects of AM251 seen in Experiments 7 and 8. For example, intra-BLA infusions of CB_1 receptor agonists induce behavioural anxiety (Rubino et al., 2008a), but attenuate stress-induced corticosterone secretion (Hill et al., 2009). Moreover, local administration of AM251 into the BLA, like i.c.v. administration (Exp. 7B and 8B), induced corticosterone secretion irrespective of stress exposure (Hill et al., 2009). As such, it is possible that AM251 acted within the BLA to interfere with the behavioural anxiety induced by CRF and cocaine withdrawal, and simultaneously stimulate corticosterone release.

Further correlative support for a potential role of the BLA in the present behavioural findings comes from a c-fos immunohistochemistry study by Patel and colleagues (2005b). In this study, systemic administration of the CB_1 receptor antagonist, SR141716, was found to both induce c-fos expression in the BLA, and to attenuate BLA c-fos expression induced by exposure to restraint stress (Patel et al., 2005b). These findings parallel those of Experiments 7A and 8A,

in which AM251 both induced behavioural anxiety, and interfered with the behavioural anxiety induced by i.c.v. CRF and cocaine withdrawal. Importantly, c-fos expression within the BLA has been shown to correlate with anxiety-like behaviour in response to pharmacological and physical stressors (Hale et al., 2010; Hsu et al., 2007).

Comparable to the mechanism proposed in Chapter 4 for reinstatement and sensitization, AM251 may counteract CRF-induced anxiety by a local interaction of the two agents within the BLA. Activation of CRF_1 receptors on BLA projection neurons has been shown to potentiate the response of these cells to excitatory input (Ugolini et al., 2008), suppress their response to inhibitory input (Rainnie et al., 2004), and increase their intrinsic excitability (Giesbrecht et al., 2010), thus promoting anxiety-like behaviour (Sajdyk et al., 1999; 2004; Rainnie et al., 2004). Many of the inhibitory inputs onto BLA projection neurons also express CB_1 receptors that, when activated by eCBs, suppress GABA release onto these neurons, increasing their excitability (Katona et al., 2001; Azad et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011). Accordingly, CB_1 receptor agonists (Rubino et al., 2008a) and $GABA_A$ receptor antagonists (Sanders and Shekhar, 1995) both induce anxiety when infused into the BLA. Taken together, these findings suggest that i.c.v. CRF may act within the BLA to activate its projection neurons and induce anxiety, and that AM251 may counter this effect by blocking eCB-mediated disinhibition of these same neurons.

In a similar manner, CB_1 receptor-mediated regulation of BLA neurons projecting to the CeA may be responsible for the effects of AM251 on cocaine withdrawal-induced anxiety. Excitatory input from the BLA to the CeA has been implicated in the regulation of many behavioural responses to stress, including drug withdrawal (Davis, 1997; Millan et al., 2003; Walker al., 2009). For example, the aversive and anxiety-like states observed during morphine withdrawal have been shown to be abolished by bilateral lesion of the BLA (Watanabe et al., 2002a), and glutamate receptor antagonism in the CeA (Watanabe et al., 2002b; Harris et al.,

2006). By disinhibiting BLA projection neurons that innervate the CeA (Katona et al., 2001; Azad et al., 2004; Zhu and Lovinger, 2005; Yoshida et al., 2011), CB_1 receptor transmission is thought to reduce the threshold for stress to activate the BLA-CeA pathway (Katona et al., 2001; Patel et al., 2005b). In support of this view, systemic pharmacological manipulations that increase eCB signaling have been shown to potentiate neuronal activation in the BLA and CeA in response to restraint stress (Patel et al., 2005b). Taken together, these findings suggest that AM251 may attenuate withdrawal-induced activation of BLA neurons projecting to the CeA, and thus interfere with cocaine withdrawal-induced anxiety.

5.6.6.2. Potential Role for Dopamine in the Effects of AM251 on CRF- and Withdrawal-induced Anxiety

CRF and DA interact within the extended amygdala to mediate behavioural anxiety. First, both the BNST and CeA contain DA terminals that make direct synaptic contact with CRF neurons (Phelix et al., 1994; Eliava et al., 2003), and DA within these regions positively regulates CRF mRNA (Day et al., 2002) and peptide levels (Stewart et al., 2008). In turn, VTA DA neurons, innervated by CRF projections from the BNST and CeA (Rodaros et al., 2007), show an increased firing rate in response to *in vitro* CRF application (Wanat et al., 2008). Accordingly, i.c.v. CRF administration (Matsuzaki et al., 1989) and exposure to stress (Herman et al., 1982; Inglis and Moghaddam, 1999) both increase amygdalar DA transmission. Behaviorally, intra-amygdala infusions of DA D_1-like receptor agonists and antagonists elicit anxiogenic and anxiolytic effects, respectively (de la Mora et al., 2010; Bananej et al., 2011), and intra-BNST administration of the D_1-like receptor antagonist, SCH23390, blocks CRF-enhanced startle responses (Meloni et al., 2006). Furthermore, conditioned place aversion produced by morphine withdrawal, an effect mediated within the extended amygdala (Watanabe

et al., 2002; Nakagawa et al., 2005), is prevented by pretreatment with the DA receptor antagonist, flupenthixol (Bechara et al., 1995; but see Laviolette et al., 2002).

Accordingly, AM251 may interfere with CRF- and cocaine withdrawal-induced anxiety via its influence on DA. As discussed in Chapter 4, eCBs released from VTA DA neurons act on presynaptic CB_1 receptors to induce a net reduction in inhibitory tone over DA neuron activity, and thereby increase DA cell firing and release (Riegel and Lupica, 2004; Pan et al., 2008; Gessa et al., 1998; Pillolla et al., 2007). Given that amygdalar DA positively regulates CRF signaling and anxiety, it is possible that AM251 induces its anxiolytic effects in CRF-treated and cocaine-withdrawn rats by suppressing DA release in the CeA, BNST, and BLA.

5.6.6.3. Potential Non-Specific Effects of AM251 on CRF- and Withdrawal-induced Anxiety

As discussed in Chapter 4, AM251 has been shown to act as an inverse agonist at CB_1 receptors (Pertwee, 2005), and an antagonist at adenosine A_1 receptors (Savinainen et al., 2003). The possibility that AM251 reverses CRF- and cocaine withdrawal-induced anxiety by inhibiting constitutively active CB_1 receptors, independent of eCB activity, cannot be ruled out without clear evidence that these stressors do in fact mobilize eCBs. However, inverse agonism is unlikely to account for the present findings, given that the behavioural effects of AM251 were most potent in combination with CRF and during cocaine withdrawal, rather than under basal conditions when activity-dependent mobilization of eCBs would presumably be lowest, and the sensitivity of CB_1 receptors to inverse agonism would presumably be most pronounced. Any blockade of adenosine A_1 receptors by AM251 is also unlikely to have contributed to its anxiolytic effects on CRF- and cocaine withdrawal-induced anxiety, given that adenosine A_1 receptor antagonists have been shown to be without effect on basal (Jain et al., 1995) or ethanol withdrawal-induced anxiety-like behaviour (Prediger et al., 2006), and to intensify symptoms of

benzodiazepine withdrawal (Listos et al., 2006). On the other hand, adenosine A_1 receptor agonists are generally anxiolytic (Jain et al., 1995; Kulkarni et al., 2007), and have been shown to attenuate ethanol withdrawal-induced anxiety (Prediger et al., 2006) and somatic benzodiazepine withdrawal (Listos et al., 2005).

5.6.6.4. Role for Non-CB_1 Receptors in the Effects of eCBs on Behavioural Anxiety

eCBs have been shown to modulate anxiety through receptor systems other than CB_1, most notably CB_2 receptors and transient receptor potential vanilloid type 1 (TRPV1) channels. In the brain, CB_2 receptors are sparsely expressed on postsynaptic elements in similar regions to CB_1 receptors, such as the hippocampus, olfactory bulb, neocortex, amygdala, striatum, thalamus and cerebellum (Gong et al., 2006; Onaivi et al., 2006). TRPV1 channels are expressed in high density in the cortex, hippocampus, and PAG (Cristino et al., 2006; 2008; McGaraughty et al., 2003; Mezey et al., 2000; Tóth et al., 2005), occasionally within the same neurons or synapses as CB_1 receptors (Cristino et al., 2006; Maione et al., 2006; Micale et al., 2009). Evidence to date suggests that both CB_2 receptors and TRPV1 channels modulate behavioural anxiety in a manner opposite to that of CB_1 receptors. For example, Onaivi and colleagues (2008) demonstrated that acute administration of the CB_2 receptor agonist, LWH015, increased anxiety-like behaviour in mice in the light-dark transition box, whereas i.c.v. administration of an antisense oligonucleotide sequence directed against CB_2 receptor mRNA reduced anxiety-like behaviour. Similarly, the TRPV1 antagonist, capsazepine, when administered systemically (Kasckow et al., 2004) or locally into the dorsolateral PAG (Terzian et al., 2009), ventral hippocampus (Santos et al., 2008), or mPFC (Aguiar et al., 2009), reduced anxiety-like behaviour in the EPM. While the potential relevance of eCB signaling at CB_2 receptors and TRPV1 channels to the present findings is currently unclear, it warrants

mentioning that the role of eCBs in anxiety is more complex than their action at CB_1 receptors alone.

CHAPTER 6

General Discussion

CHAPTER 6

General Discussion

The series of experiments presented in this dissertation examined the modulatory actions of two neurochemical systems, TCAP-1 and eCBs, on the expression of cocaine- and anxiety-related behaviours induced or mediated by the stress peptide, CRF. The present findings extend earlier reports of the regulatory actions of TCAP-1 on anxiety-like behaviours to show for the first time that TCAP-1 potently and selectively interferes with the actions of CRF on cocaine-related behaviours. Specifically, repeated treatment with TCAP-1 blocked the effects of CRF on the reinstatement of cocaine seeking and expression of cocaine-sensitized locomotion; the same TCAP-1 treatment regimen was, however, without effect on cocaine- and footshock-induced reinstatement of cocaine seeking, and was also without effect on the expression of cocaine-sensitized locomotion elicited by a cocaine challenge. The present experiments provide further novel evidence of a functional interaction between eCB and CRF systems. Specifically, acute pretreatment with the CB_1 receptor antagonist, AM251, interfered in the expression of CRF-, but not footshock- or cocaine-, induced reinstatement of cocaine seeking, as well as in the expression of cocaine-sensitized locomotion to challenge injections of both CRF and cocaine. Furthermore, AM251 pretreatment reversed behavioral anxiety induced by CRF and withdrawal from repeated cocaine administration, in a manner that did not parallel its effect on plasma corticosterone; these results suggest that the anxiogenic effects of CRF and cocaine withdrawal are mediated by CB_1 receptor transmission, independent of HPA axis regulation.

One striking feature of the present findings is the similarity with which administrations of TCAP-1 and AM251 modulated the behaviours under investigation. Although this is the first set of studies to show such striking parallels in the roles that these systems play in cocaine-related behaviours, the findings are consistent with a growing body of literature pointing to similar roles for TCAP-1 and eCBs in cellular and behavioural responses more generally.

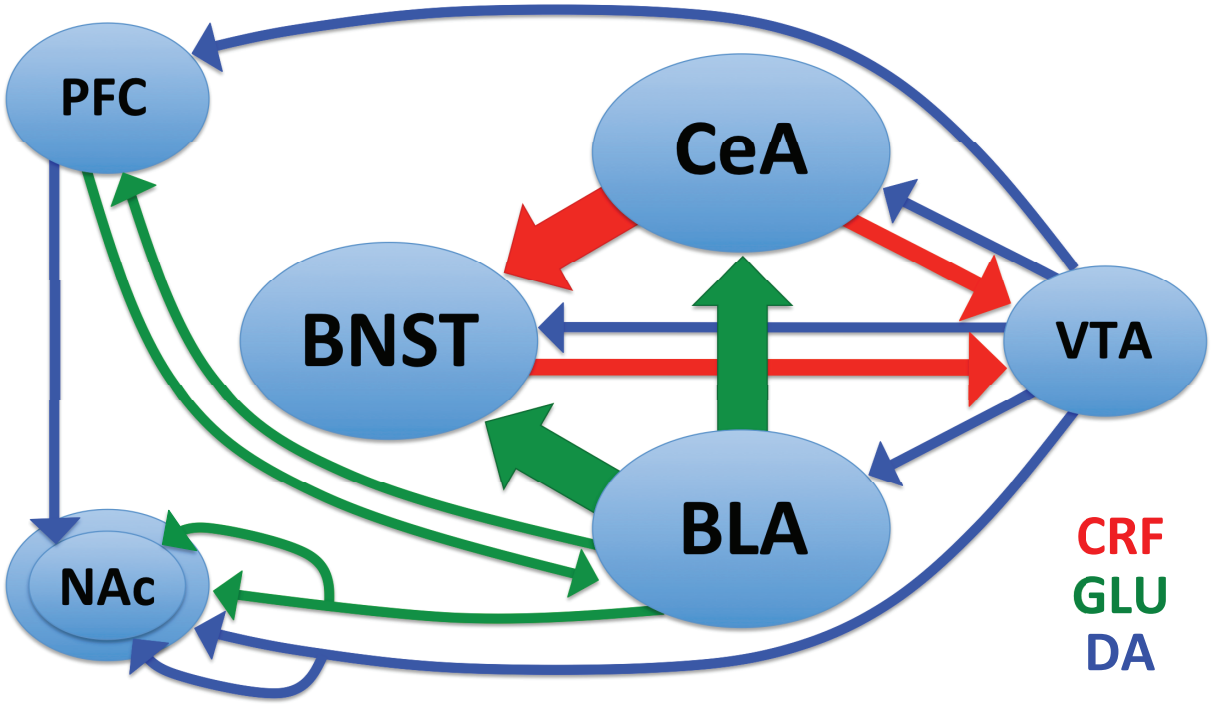
Indeed, the pronounced regional overlap in the central distribution of TCAP-1 and CB_1 receptors (Tsou et al., 1998; Wang et al., 2005b; Torres et al., 2011), and their shared regulation of various cellular and behavioural responses to stress (Trubiani et al., 2007; Nagayama et al., 1999; Al Chawaf et al., 2007b; Rubino et al., 2008a), provided the primary rationale for the present parallel investigation of the two systems.

6.1. Toward a Model of CRF-TCAP-eCB Interactions in the Mediation of Cocaine- and Anxiety-Related Behaviours

The novel findings reported on, and the literature reviewed, in the present dissertation contribute to an emerging model of CRF-TCAP-eCB interactions in the mediation of cocaine- and anxiety-related behaviours. This model, developed below, emphasizes TCAP-1 and eCB interactions with CRF within a circuitry that includes interconnections between the CeA, BNST, and BLA, and that extends to, and is reciprocally innervated by, the mesocorticolimbic DA system. Some of the essential neurochemical pathways comprising this circuitry are depicted in Figure 34.

First, the fact that TCAP-1 and AM251 exert similar effects on a diverse set of cocaine-related behaviours suggests, at the least, a degree of functional relatedness between the systems mediating these behaviours. Indeed, although reinstatement of cocaine seeking, cocaine-induced behavioural sensitization, and cocaine withdrawal-induced behavioural anxiety are not thought to have identical neuronal substrates, there is clearly overlap in the neuroanatomical and neurochemical systems mediating these different behaviours (Erb et al., 2010; Steketee and Kalivas, 2011; Walker et al., 2009). Most emphasized in this dissertation is the shared engagement and responsivity of these behaviours to central CRF signaling, the likely involvement of the CeA, BNST, and BLA in these effects of CRF, and the functional

Figure 34



Schematic depicting the relevant neurocircuitry involved in the proposed CRF-TCAP-eCB interaction.

Abbreviations: Basolateral amygdala (BLA), bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), corticotropin-releasing factor (CRF), dopamine (DA), glutamate (GLU), nucleus accumbens (NAc), prefrontal cortex (PFC), ventral tegmental area (VTA).

interactions between CRF and other neurochemical systems within these regions, such as DA, noradrenaline, glutamate, and GABA systems (Erb et al., 2010; Steketee and Kalivas, 2011; Tyacke et al., 2010).

In developing a model of CRF-TCAP-eCB interactions, it is important to note that the positive behavioural effects reported on in this dissertation were obtained following *repeated* administration of TCAP-1 and *acute* administration of the CB_1 receptor antagonist, AM251. The efficacy of TCAP-1 and AM251 obtained through these different regimens likely reflects differences in their fundamental mode of action. Indeed, evidence suggests that TCAP-1 modifies behaviour by inducing long-lasting changes in intracellular signaling and cell structure, and that repeated exposures to the peptide are required for these changes to be manifest (Al Chawaf et al., 2007a; b; De Almeida et al., 2011; Chand et al., 2011; Tan et al., 2011). On the other hand, AM251 exerts its acute behavioural effects by transiently interfering with eCB-mediated suppression of neurotransmitter release, particularly that of glutamate and GABA (Szabo and Schlicker, 2005). It warrants mention, however, that repeated exposure to CB_1 receptor antagonists has been shown to induce persistent changes in behaviours comparable to those studied in this dissertation. For example, Gerdeman and colleagues (2008) showed that the sensitized locomotor response to a cocaine challenge seen in cocaine pre-exposed mice was abolished by repeated (5-day) systemic treatment with the CB_1 receptor antagonist, SR141716, in the previously cocaine-paired environment (Gerdeman et al., 2008). Furthermore, 14-day treatment with SR141716 during chronic mild stress abolished the subsequent stress-induced increase in anxiety-like behaviour in the EPM and depression-like immobility in the forced swim test (Griebel et al., 2005). From this perspective, an important line of future research would be to investigate whether repeated pre-exposure to AM251 interferes with cocaine- and anxiety-related behaviours in a manner similar to acute pretreatment.

The impetus for a model of CRF-TCAP-eCB interactions stems largely from the potent effects of both TCAP-1 and AM251 on i.c.v. CRF-induced behaviours. At the cellular level, it is plausible that repeated TCAP-1 exposure produces long-lasting changes in the intracellular signaling and/or structure of CRF receptor-expressing cells to modify the effects of subsequent CRF treatment (Al Chawaf et al., 2007a; b; Tan et al., 2008; De Almeida et al., 2011; Chand et al., 2011). In the case of acute AM251 treatment, antagonism of CB_1 receptors may transiently disrupt the balance of excitatory and inhibitory transmission onto CRF receptor-expressing neurons, thus modifying their responsivity to the neuropeptide (Szabo and Schlicker, 2005; Hermann and Lutz, 2005; Ramikie and Patel, 2011).

At the regional level, it is known that both TCAP-1 and CB_1 receptors are expressed to varying degrees in CRF-rich brain structures, including those comprising the extended amygdala and BLA. More specifically, TCAP-1 expression is especially prominent in the BLA and CeA, and, albeit to a lesser degree, is also found in the BNST (Wang et al., 2005b; Torres et al., 2011; Chand et al., unpublished). CB_1 receptors, on the other hand, are densely expressed in the BLA, moderately expressed in the BNST, and barely detectable in the CeA (Herkenham et al., 1991; Marsicano and Lutz, 1999; Katona et al., 2001). Intracerebroventricular CRF has been shown to activate each of these regions (Bittencourt and Sawchenko, 2000), and CRF transmission within these regions has been implicated in the expression of reinstatement, sensitization, and anxiety behaviours (Erb and Stewart, 1999; Richter et al., 1995; Erb et al., 2005; Sajdyk et al., 1999; Sahuque et al., 2006). Taken together, this evidence supports the view that TCAP-1 and eCBs may regulate the responsivity of CRF receptor-expressing cells within the extended amygdala and BLA to influence the effects of i.c.v. CRF on cocaine- and anxiety-related behaviours.

The BNST has been identified as an important neuronal substrate mediating the effects of i.c.v. CRF on reinstatement of cocaine seeking (Erb and Stewart, 1999). As such, it is possible that TCAP-1 and AM251 act directly within the BNST to counter this effect of CRF,

by modulating the responsivity of local CRF receptor-expressing neurons; this modulation may occur via changes in intracellular signaling/structure or afferent neurotransmission. Alternatively, or in addition, TCAP-1 and AM251 may modulate CRF-induced reinstatement through their actions in the BLA. Indeed, BLA projection neurons are activated by CRF (Ugolini et al., 2008; Giesbrecht et al., 2010), and provide an important source of excitatory input to the BNST (Dong et al., 2001; Sah et al., 2003; Walker et al., 2009). TCAP-1 may suppress the intracellular response of BLA projection neurons to CRF signaling; in addition, AM251, by blocking the eCB-mediated suppression of GABA release onto BLA projection neurons (Katona et al., 2001; Azad et al., 2004; Yoshida et al., 2011), may counteract the excitatory effects of CRF on these neurons. In this manner, TCAP-1 and AM251 may act within the BNST and BLA to modulate CRF-induced reinstatement.

The cocaine-sensitized locomotor response to a CRF challenge has been found to correlate with enhanced neuronal activation in the CeA (Erb et al., 2003; 2005); thus, it is possible that CRF transmission within the CeA mediates the expression of cocaine sensitization. As such, TCAP-1 may act directly within the CeA to suppress the cellular responsivity to i.c.v. CRF. A similarly direct action of AM251 is unlikely, however, given the barely detectable expression of CB_1 receptors within the CeA (Katona et al., 2001). On the other hand, expression patterns of TCAP-1 and CB_1 receptors suggest that TCAP-1 and AM251 may *indirectly* modulate CeA activity via their action in the BLA. It is possible, that is, that TCAP-1 suppresses the CRF-induced responsivity of BLA projections that innervate the CeA, and that AM251 enhances the inhibitory tone on these same neurons. In this way, both TCAP-1 and AM251 could modify activation of the CeA in response to i.c.v. CRF, and thereby interfere with the expression of the sensitized locomotor response.

The anxiety-like behaviour induced by i.c.v. CRF administration is blocked by pharmacological inactivation of the BLA (Walker et al., 2009) and local infusion of CRF

receptor antagonists into the BNST (Lee and Davis, 1997). Furthermore, infusions of CRF into either structure induce anxiety-like behaviour (Sajdyk et al., 1999; Sahuque et al., 2006). As previously mentioned, both structures also exhibit notable TCAP-1 and CB_1 receptor expression. Functionally, TCAP-1 into the BLA modulates anxiety-like behaviour (Wang et al., 2005b), and CB_1 receptor agonists into the BLA induce anxiety (Rubino et al., 2008a). Taken together, it would seem reasonable to speculate that TCAP-1 and AM251 reduce the responsivity of neurons in the BLA (and possibly BNST) to CRF treatment, and thereby interfere with CRF-induced anxiety.

The present effects of AM251, unlike TCAP-1, extend beyond those induced by i.c.v. CRF to include the blockade of cocaine-induced expression of sensitization and cocaine withdrawal-induced anxiety. This additional efficacy of AM251 could be explained by the specific brain substrates underlying these behaviours. Indeed, consistent with other behaviours effectively modified by AM251, cocaine-induced expression of sensitization and withdrawal-induced anxiety are both associated with heightened CRF transmission within the CeA (Richter et al., 1995; 1999; Maj et al., 2003), and are both reliant on intact BLA function (Kalivas and Alesdatter, 1993; Harris et al., 2006). Moreover, the inability of AM251 to interfere with both footshock- and, in the present work, cocaine-induced reinstatement of cocaine seeking, is in line with evidence that neither condition for reinstatement recruits CRF transmission in the CeA (Erb and Stewart, 1999; McFarland and Kalivas, 2001), nor is either dependent on the BLA (McFarland et al., 2004; McFarland and Kalivas, 2001). From this perspective, however, it is of interest that TCAP-1, which is expressed in the BLA (Wang et al., 2005b; Torres et al., 2011), and thought to act within the BLA to modify other cocaine-related behaviours, was ineffective in blocking cocaine-induced expression of sensitization. This null effect underscores the notion that the ideal conditions for detecting behavioural effects of TCAP-1 treatment may be the widespread engagement of CRF receptors by i.c.v. CRF administration (Al Chawaf et al.,

2007b; Tan et al., 2008; 2009). Moreover, this discrepancy between the effects of TCAP-1 and AM251 may be attributed to the ability of CB_1 receptor antagonists like AM251 to constrain VTA DA neuron activity (French, 1997; Gessa et al., 1998; Cheer et al., 2003), which may contribute to their ability to interfere with a more diverse set of cocaine-related behaviours.

The mesocorticolimbic DA system, through its extensive and reciprocal connectivity with central CRF systems, is likely to play an important role in the expression of CRF-TCAP-eCB interactions. Indeed, CRF input to the VTA, presumably from the CeA and BNST (Rodaros et al., 2007), increases the excitability of VTA DA neurons (Ungless et al., 2003; Wanat et al., 2008), and promotes DA transmission in terminal regions such as the PFC, NAc, and amygdala (Lavicky and Dunn, 1993; Matsuzaki et al., 1998; Dunn, 2000). In turn, DA projections synapse directly onto CRF neurons within the CeA (Eliava et al., 2003) and BNST (Phelix et al., 1994), stimulate local CRF transmission (Day et al., 2002; Stewart et al., 2008; Kash et al., 2008), and drive activation of the extended amygdala (Bittencourt and Sawchenko, 2000; Kash et al., 2008). Similarly, DA has been shown to act in the amygdala to potentiate (Krishnan et al., 2011), and mediate CRF-potentiated (Krishnan et al., 2010), excitatory input from the BLA to the CeA in cocaine pre-exposed animals.

CRF and DA systems also functionally interact to mediate various cocaine- and anxiety-related behaviours. For example, CRF-induced activation of mesocortical DA projections has been widely implicated in the reinstatement of cocaine seeking (Wang et al., 2005a; 2007; McFarland et al., 2004), and anxiety-like behaviour (Refojo et al., 2011), whereas CRF-induced activation of DA projections to the NAc is thought to play an important role in locomotor sensitization (Kalivas et al., 1983; Lu et al., 2003; Hahn et al., 2009). Similarly, DA signaling within the amygdala, seen in response to stress and CRF treatment (Herman et al., 1982; Inglis and Moghaddam, 1999; Matsuzaki et al., 1989; Yokotama et al., 2005), has been shown to positively regulate cue-induced reinstatement of cocaine seeking (See et al., 2001; Ledford et

al., 2003) and anxiety (de la Mora et al., 2010; Meloni et al., 2006), and mediate the development of locomotor sensitization to amphetamine (Bjijou et al., 2002). In light of the many neuroanatomical and functional interactions of CRF and DA systems, it is plausible that the VTA serves as a common site of action for TCAP-1 and eCBs to regulate behaviours induced or mediated by CRF. Indeed, TCAP-1 and AM251 may act in the VTA, in addition to the extended amygdala and BLA, to suppress the responsivity of DA neurons to CRF, and thereby interfere with cocaine- and anxiety-related behaviours.

Taken together, these findings provide the basis for an overarching framework by which to understand the actions of TCAP-1 and AM251 on cocaine- and anxiety-related behaviours that are induced or mediated by CRF. Specifically, it is proposed that TCAP-1 and AM251 act in a parallel manner to modulate CRF transmission within the BLA, CeA, BNST, and possibly VTA, and, in turn, regulate the expression of cocaine- and anxiety-related behaviours. TCAP-1 likely does so by inducing long-term changes in intracellular signaling and cellular structure of CRF receptor-expressing neurons, whereas AM251 appears to exert its modulatory effects by transiently disrupting the balance of excitatory and inhibitory neurotransmission onto CRF receptor-expressing neurons.

6.2. Limitations and Future Directions

Although the present findings reveal functional interactions of TCAP-1 and eCBs with central, presumably extrahypothalamic, CRF systems, they do not allow conclusions to be made about the loci of these interactions. As such, the next step in this research would be to conduct a series of localization studies aimed at manipulating CRF, TCAP-1, and eCB signaling within specific regions of interest (e.g. CeA, BNST, BLA, VTA), to further probe the neurobiological substrates of their interactions.

Given the excitatory effects of CRF on DA neuron activity and release (Ungless et al., 2003; Wanat et al., 2008; Matsuzaki et al., 1989; Lavicky and Dunn, 1993), the capacity for CB_1 receptor antagonists to constrain this activity and release (French, 1997; Gessa et al., 1998; Cheer et al., 2003), and the established role of DA transmission in many of the behaviours under investigation in this dissertation (Brown et al., unpublished; Steketee et al., 2011; Meloni et al., 2006; Bechara et al., 1995), future studies should be conducted to clarify the potential role of downstream DA release in the effects of AM251, and even TCAP-1, on CRF-induced behaviours. First, a series of *in vivo* microdialysis studies could be performed to examine whether or not AM251 pretreatment or TCAP-1 pre-exposure reduces CRF-induced DA release in the PFC, NAc, BNST, CeA, or BLA. The functional significance of these findings could subsequently be examined through a series of localization studies in which various DA receptor agents are locally infused into the same regions; specifically, studies could test whether local D_1-like antagonists are capable of blocking CRF-induced behaviours, and whether D_1-like receptor agonists are capable of rescuing CRF-induced behaviours inhibited by prior treatment with AM251 or TCAP-1.

One important limitation of the work presented in Chapters 4 and 5 is the exclusive use of CB_1 receptor antagonism to examine the role of eCB signaling in cocaine- and anxiety-related behaviours. This 'loss of function' approach to studying eCB signaling should, in future, be complemented by the use of eCB uptake/degradation inhibitors to assess whether potentiated eCB signaling has effects opposite to those induced by AM251. Bidirectional analysis of this kind is particularly important in light of paradoxical evidence that systemic eCB reuptake inhibitors and CB_1 receptor antagonists have both been shown to attenuate nicotine- and cue-induced reinstatement of nicotine seeking (Forget et al., 2009; De Vries et al., 2005), block the development of amphetamine sensitization (Eisenstein et al., 2009; Corbillé et al., 2007), and reduce anxiety on the EPM (Patel and Hillard, 2006; Griebel et al., 2005).

This dissertation is similarly limited by its use of a single CB_1 receptor antagonist. Despite the high selectivity of AM251 for the CB_1 receptor (Ashton et al., 2008), the present findings would be strengthened by the use of additional CB_1 receptor antagonists, as well as pharmacological agents (e.g. adenosine A_1 receptor agonists) to rule out any non-specific effects of these CB_1 receptor antagonists.

Understanding the effects of TCAP-1 on CRF action will ultimately require the definitive characterization and localization of the TCAP-1 binding site. Moreover, despite the considerable evidence demonstrating that TCAP-1 functionally interacts with CRF to mediate stress-related behaviours, the corresponding anatomical and physiological data are lacking. Future studies using double-label immunohistochemistry and *in situ* hybridization to co-localize TCAP-1 (and, ultimately, its binding sites) with CRF and CRF receptor protein and mRNA would help to clarify the nature of TCAP-CRF interactions at the cellular level. Furthermore, *in vitro* electrophysiological analysis of the effects of TCAP-1 application on CRF-modulated excitatory and inhibitory transmission in BLA and CeA neurons, for example, represents an important focus for future research.

Similar neuroanatomical and physiological studies need to be performed to probe the nature of eCB-CRF interactions. In this case, double-label immunohistochemistry with electron microscopy is necessary to definitively determine whether CRF-immunoreactive axon terminals co-express CB_1 receptors. Such studies, paired with others investigating the effects of eCBs on the electrophysiological response to endogenously generated and exogenously applied CRF, would provide important clarification as to whether or not CB_1 receptor activation is capable of modulating CRF release or neurotransmission.

Another important line of future research is to investigate whether TCAP-1 and eCB systems interact to modulate CRF-induced behaviours. To probe the nature of such an interaction, studies should be conducted that involve combining TCAP-1 pre-exposure and

AM251 pretreatment regimens that show partial efficacy – that is, regimens that, when administered individually, only partially attenuate the reinstatement, sensitized locomotion, and anxiety induced by CRF. If, under these conditions, the modest inhibitory effects of TCAP-1 and AM251 summate, one may deduce that TCAP-1 and eCB systems act independently to modulate the behavioural effects of CRF. Alternatively, if TCAP-1 pre-exposure occludes any further inhibition by a low dose pretreatment of AM251, TCAP-1 and eCB systems may share a common regulatory mechanism over CRF-induced behaviour. Furthermore, future studies that characterize the cellular colocalization of TCAP-1 and CB_1 receptors could provide important insight into the shared cellular and behavioural functions of the TCAP-1 and eCB systems.

To further probe the generality of the present effects of TCAP-1 and AM251 administration on the reinstatement of cocaine seeking, future studies should be conducted using different stressors and behavioural paradigms. For example, exposure to acute food deprivation stress, and administration of the pharmacological stressor, yohimbine, have been shown to induce the reinstatement of morphine (Shalev et al., 2006) and alcohol seeking (Marinelli et al., 2007) in a CRF-dependent manner. Thus, extending the present work to investigate the role of TCAP-1 and eCBs in the reinstatement of cocaine seeking induced by these or other stressors would seem an important question for future research (but see Brown et al., 2009). Similarly, studies investigating a role for TCAP-1 and eCBs in stress-induced reinstatement using alternate behavioural paradigms, such as CPP (Lu et al., 2001), would contribute in an important way to a more thorough characterization of the involvement of these systems in the neurobiology and phenomenology of reinstatement.

The cross-sensitization between stress and cocaine has received comparatively limited research attention to date, and other than the present findings, the role of TCAP-1 and eCBs in cross-sensitization is wholly uncharacterized. One critical next step in this research would be to assess whether the effects of TCAP-1 and AM251 on the cross-sensitization between cocaine

and CRF generalize to sensitization paradigms using behavioural stressors, such as the cocaine-sensitized locomotor response to restraint stress (Maeda et al., 2009). On the other hand, paradigms such as social defeat stress-induced locomotor sensitization to cocaine (Boyson et al., 2011), and forced swim stress-facilitated acquisition of cocaine CPP (Kreibich et al., 2009), would be useful to investigate whether TCAP-1 and eCB systems play a similar role in stress-induced cross-sensitization to cocaine as they do in cocaine-induced cross-sensitization to stress.

Another important question for future research would be to determine whether manipulations of TCAP-1 and eCB systems modulate changes in cocaine self-administration induced by CRF or other stressors. Based on reports that cocaine self-administration is facilitated by footshock (Goeders and Guerin, 1994) and social defeat stress (Boyson et al., 2011), and attenuated by CRF_1 receptor antagonists (Goeders and Guerin, 2000; Specio et al., 2007), experiments were in fact planned to address this question in the present dissertation. More specifically, two parallel sets of experiments were designed to study the effects of repeated TCAP-1 and acute AM251 administration on i.c.v. CRF-induced modulation of cocaine self-administration. These studies were not successfully executed, however, due to a failure in initial pilot work to establish that i.c.v. CRF modulates cocaine self-administration. Thus, future studies aimed at carrying out a more complete parametrical assessment of the effects of i.c.v. CRF on cocaine self-administration, and the effects of TCAP-1 and AM251 on the modulation of cocaine self-administration by other stressors (e.g., footshock, social defeat stress) is warranted.

Finally, a logical extension to the present findings of a role for TCAP-1 in the effects of CRF on cocaine-related behaviours, and to previous work by Lovejoy and colleagues demonstrating that TCAP-1 regulates CRF-induced anxiety (Al Chawaf et al., 2007b; Tan et al., 2008), would be to examine the effects of TCAP-1 on the CRF-dependent behavioural anxiety induced by cocaine withdrawal.

6.3. Clinical Implications

Clinical studies indicate a complex, multifaceted relationship between stress and cocaine abuse in humans. Indeed, heightened states of anxiety experienced during initial cocaine withdrawal often impede abstinence in regular cocaine users (Gawin and Kleber, 1986; Bradley et al., 1989). Moreover, following prolonged drug-free periods, cocaine abusers show heightened responsivity to stress that is thought to contribute to their risk for relapse following a stressful experience (Brady et al., 2009; Chaplin et al., 2010; Sinha et al., 2006; Back et al., 2010). As such, effective treatment programs for cocaine addiction, although elusive to date, are likely to include pharmacological interventions that help manage acute withdrawal symptoms and normalize long-term stress responsivity (Kampman, 2010).

As highlighted in this dissertation, preclinical studies point to a critical role for central CRF signaling in the experience of withdrawal-induced anxiety, the sensitized responsivity to stress following cocaine exposure, and the ability of stress to trigger relapse. Accordingly, considerable interest exists in developing pharmacological treatments that target CRF systems. In a recent Phase II clinical trial, the non-peptide CRF_1 receptor antagonist, R121919, showed promise in alleviating depressive and anxiety-related symptoms in patients with major depression (Zobel et al., 2000); however, these trials were recently discontinued due to reports of elevated liver enzymes in participants (Zorrilla and Koob, 2010). Clinical trials for other novel non-peptide CRF_1 receptor antagonists in the treatment of major depression and social and generalized anxiety disorders (Zorrilla and Koob, 2010) have since been initiated. The success of these drugs in effectively treating stress-related disorders will be critical in determining the clinical evaluation of CRF_1 receptor antagonists for cocaine addiction.

The TCAP-1 and eCB systems, shown here to functionally interact with CRF in anxiety- and cocaine-related behaviours, may be promising targets in the development of treatments for stress-related conditions, and by extension, addictive disorders. TCAP-1 is currently

undergoing preclinical development by Protagenic Therapeutics Inc. as a potential antidepressant, anxiolytic, and neuroprotective agent (PT00114). Building on findings from Experiment 2 of this dissertation, testing is currently underway to investigate whether TCAP-1, when given through the more clinically relevant i.v. route of administration, retains its efficacy in blocking CRF-induced reinstatement of cocaine seeking in rats. Pending positive findings in these preclinical studies, clinical trials may be undertaken to study the efficacy of TCAP-1 in treating substance dependence. In the case of CB_1 receptor antagonists, clinical trials are comparatively more advanced, but have been met with limited success. For example, clinical testing for obesity (Van Gaal et al., 2005) and cardiovascular disease (Topol et al., 2010) with Rimonabant (SR141716), the prototypical CB_1 receptor antagonist, has been discontinued due to an increased prevalence of depression and anxiety in the test groups (Christensen et al., 2007). Paradoxically, however, before being withdrawn from the European drug market, Rimonabant showed some efficacy in reducing symptoms of depression and anxiety in overweight patients with schizophrenia (Kelly et al., 2011). Altogether, the future clinical utility of TCAP-1 and CB_1 receptor antagonists is uncertain, but the present findings are in support of their preclinical development as modulators of stress-related behaviour.

6.4. Conclusion

The experiments comprising this dissertation are among the first to investigate functional interactions of TCAP-1 and eCBs with CRF in mediating cocaine- and anxiety-related behaviours. Further exploration of the nature of these interactions has important implications for understanding the regulatory mechanisms of the central stress response, as well as for characterizing potential target systems that may interfere with some of the harmful behavioural manifestations of stress in drug-dependent individuals.

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