A Study of Corticotropin-Releasing Factor-Catecholamine Interactions in the Reinstatement of Cocaine Seeking in Rats

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate in Philosophy

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Abstract

It has been well established that the stress-related neurochemical systems corticotropinreleasing factor (CRF), noradrenaline (NA), and dopamine (DA) mediate stress-induced reinstatement of drug seeking. The three series of experiments presented in this dissertation constitute a further exploration of the role these neurochemical circuits play in reinstatement by providing the first direct exploration of whether central CRF and catecholamine (NA and DA) systems interact to influence reinstatement of cocaine seeking.

The primary objective of the first series of experiments was to determine whether NA and CRF systems interact to mediate reinstatement of cocaine seeking and, if so, to determine the direction of this interaction. Results showed that central administration of NA induced reinstatement and up-regulated the expression of *c-fos* mRNA, a marker of neuronal activation, in brain regions involved in footshock-induced reinstatement. Pretreatment with a CRF antagonist blocked NA-induced reinstatement. In contrast, pretreatment with the α_2 adrenoceptor agonist, clonidine, failed to block CRF-induced reinstatement. Taken together, these findings suggest a functional interaction between NA and CRF systems in mediating stress-induced reinstatement of cocaine seeking, whereby activation of CRF receptors occurs subsequent to, and downstream of, the sites of action of NA.

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A second series of experiments examined the role of D_1 - and D_2 -like receptors in CRFinduced reinstatement. Pretreatment with the D_1 - or D_2 -like receptor antagonists, SCH23390 and raclopride, respectively, dose-dependently blocked CRF-induced reinstatement of cocaine seeking. Taken together with previous findings, these results suggest that CRF-induced reinstatement of cocaine seeking likely involves DAergic signaling via D_1 - and D_2 -like receptors, subsequent to activation of CRF receptors.

The final series of experiments investigated the neuropharmacology of yohimbineinduced reinstatement, focusing on the roles of α_2 -adrenoceptors, D₁- and D₂-like receptors. These experiments were prompted by an unexpected finding in the first series of experiments, in which a CRF antagonist failed to interfere in yohimbine-induced reinstatement of cocaine seeking. Results showed that pretreatment with the α_2 -adrenoceptor agonist, clonidine, or raclopride, prior to tests for yohimbine-induced reinstatement failed to influence responding. In contrast, pretreatment with SCH23390 blocked yohimbine-induced reinstatement. Taken together, these findings suggest that yohimbine may act through system(s) other than NA to have its effects.

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Finally, I dedicate this thesis to my family, especially my mum, Joan Brown, because this thesis would not exist without her. She has been instrumental in creating and guiding me down the path that led me to this degree, and for that I am eternally grateful.

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- Brown ZJ, Tribe E, D'souza NA, Erb S (2009) Interaction between noradrenaline and corticotrophin-releasing factor in the reinstatement of cocaine seeking in the rat. Psychopharmacology 203(1):121-30

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List of Abbreviations

6-hydroxydopamine 6-OHDA
Adrenocorticotropic hormone ACTH
α -amino-3-hydroxy-5-methyl-4-
isoxaxolepropionic acid AMPA
Bed nucleus of the stria terminalisBNST
Central nucleus of the amygdalaCeA
Conditioned place preference CPP
Corticotropin releasing factor
Dopamine
Dopamine β-hydroxylase
Excitatory post synaptic current EPSC
γ-aminobutyric acid GABA
Intracerebroventricular
Intraperitoneal i.p.
Intravenous

NoradrenalineNA
Hypothalamic-pituitary-adrenal
axis HPA axis
Locus coeruleusLC
Lateral tegmental nuclei Ltg nuclei
<i>N</i> -Methyl-D-aspartic acid
Nucleus accumbens
Medial Prefrontal Cortex mPFC
Orbital frontal cortex OFC
Prefrontal cortex
Paraventricular nucleus PVN
Serotonin (5-Hydroxytryptamine) 5-HT
Tetrodotoxin
Ventral tegmental areaVTA

CHAPTER 1

General Introduction

Chapter 1: General Introduction

Stress has long been considered an important factor contributing to drug craving and relapse to drug use in humans (Kreek and Koob, 1998). Indeed, clinical reports show that individuals with a history of drug use frequently attribute episodes of relapse to stressful life events and negative mood states (Bradley et al., 1989; Sinha, 2001). Consistent with these findings, controlled laboratory experiments designed to induce acute states of psychological distress in cocaine and alcohol dependent patients report significant increases in drug craving following exposure to this stressful state (Sinha and O'Malley, 1999; Sinha, 2009; Chaplin et al., 2010). To further investigate the factors that contribute to stress-induced relapse documented in clinical and epidemiological studies, animal models of relapse, known as reinstatement procedures, have been developed.

The reinstatement model of relapse was first developed by Stretch et al (1971) as a method for studying the effects of acute, non-contingent exposure to drugs and conditioned stimuli on the reinstatement of drug-seeking behaviour in monkeys. These procedures were subsequently extended to studies involving rats and mice, and were modified to study additional factors contributing to relapse to drug seeking, including exposure to stress.

Generally, the reinstatement procedure involves training an animal to perform an operant behaviour, such as a lever press or nose poke, for an intravenous infusion of drug reinforcement. Subsequently, the drug-reinforced behaviour is extinguished by removal of the reinforcer, such that the operant response no longer results in a drug infusion. Reinstatement of the extinguished behaviour is then induced by exposure of the animal to a non-contingent injection of the previously self-administered drug (or one like it), exposure to cues previously associated with drug taking, or exposure to acute physical, environmental or pharmacological

stressors. The number of non-reinforced operant responses made following exposure to these stimuli is used as a measure of drug-seeking behaviour (Shaham et al., 2003).

The appeal of the reinstatement model for basic scientists and clinicians derives from the observations that factors reported to reinstate drug seeking in laboratory animals also provoke relapse and craving in humans, including acute re-exposure to the previously selfadministered drug (Jaffe et al., 1989), drug associated cues (Childress et al., 1993), or stress (Sinha, 2001, 2009). From this perspective, the model is said to have high criterion validity (also referred to as predictive validity), which refers to the extent experimental manipulations of laboratory animals are predictive of conditions that effect human behaviour (Epstein et al., 2006). As such, the reinstatement model has been used extensively to study the putative neurobiological systems that mediate relapse to drug-seeking behaviour.

Stress-Induced Reinstatement

In initial experiments aimed at studying the effects of stress on reinstatement of drug seeking, laboratory rats were exposed to acute, intermittent footshock stress at the time of testing for reinstatement. Studies were first carried out in animals with histories of heroin and cocaine self-administration. In these experiments, it was found that 10-min exposure to brief, mild, and intermittent footshock robustly reinstated drug-seeking after 1-2 weeks of extinction training and again after an additional 4-6 week drug-free period (Shaham and Stewart, 1995; Erb et al., 1996). Subsequently, these findings were extended to animals with other drug histories, including alcohol, nicotine, and methamphetamine (Le et al., 1998; Buczek et al., 1999; Zislis et al., 2007; Beardsley et al., 2010), and were replicated using modified schedules of reinforcement, drug dose, footshock parameters, and rat strains (Ahmed and Koob, 1997; Mantsch and Goeders, 1999; Ahmed et al., 2000).

The effects of footshock on reinstatement of drug seeking have also been extended to the recovery of other extinguished drug-related behaviours; perhaps most notably, druginduced conditioned place preference (CPP). The CPP procedure is used to measure the unconditioned reinforcing effects of a drug developed through repeated pairings of that drug with distinct contextual cues (Bardo and Bevins, 2000). It has been shown that, following the development and subsequent extinction of morphine-induced CPP, 15-min exposure of rats to intermittent footshock stress robustly increases the time spent in the previously morphinepaired environment (Wang et al., 2001; Wang et al., 2006). Using similar parameters, this effect was subsequently extended to rats conditioned with cocaine (Lu et al., 2002).

The effect of footshock stress on reinstatement generalizes to other stressors. For example, acute, 1-day food deprivation stress, prior to tests for reinstatement, induces relapse to heroin- (Shalev et al., 2000) and cocaine- (Highfield et al., 2002; Shalev et al., 2003) seeking behaviour. Likewise, exposure to forced swim or restraint stress has been shown to reliably induce the reinstatement of an extinguished cocaine- and morphine- CPP, respectively (Kreibich and Blendy, 2004; Carey et al., 2007; Mantsch et al., 2010). In contrast, exposure to social-defeat stress, which is regarded as a more ethologically valid stressor than footshock (Miczek et al., 2008), failed to reinstate alcohol seeking (Funk et al., 2005). Likewise, whereas restraint was effective in the CPP procedure, this stressor was ineffective in inducing reinstatement of extinguished heroin seeking (Shalev et al., 2000). The discrepancy in the effect of restraint on reinstatement in the self-administration and CPP procedures may be attributed to the fact that in the self-administration procedure, physical restraint occurred outside of the self-administration chambers. In contrast, exposure of animals to restraint prior to tests for reinstatement of drug-CPP occurred inside the CPP apparatus. Indeed, Shalev and colleagues (2000) showed that exposure to footshock stress is only effective in inducing

reinstatement if it is administered in the context in which drug self-administration is experienced.

In addition to acute environmental stress, administration of pharmacological stressors has been shown to induce reinstatement. For example, intracerebroventricular (i.c.v.) injection of the stress-related neuropeptide, corticotropin releasing factor (CRF), prior to tests for reinstatement, results in relapse to drug-seeking behaviour, an effect most likely mediated via its direct action in the brain at extra-hypothalamic brain sites (Erb et al., 1998). Similarly, injections of the synthesis inhibitor of corticosterone, metyrapone, administered prior to tests for reinstatement has been shown to reinstate heroin-seeking behaviour, most likely by inducing activation of the stress axis (Shaham et al., 1997). More recently, it has been reported that the α_2 -adrenoreceptor antagonist, yohimbine, which induces stress-like responses in humans and nonhumans, reinstates drug seeking in monkeys (Lee et al., 2004) and rats (Shepard et al., 2004; Le et al., 2005; Fletcher et al., 2008; Kupferschmidt et al., 2009), as well as extinguished cocaine-induced CPP in mice (Mantsch et al., 2010).

In recent years, considerable work has been conducted to determine the neurobiological underpinnings of stress-induced, and in particular footshock-induced, reinstatement of drug seeking. A major, though not exclusive, focus of this work has been on the roles of three stress-related neurochemical systems, CRF, noradrenaline (NA), and dopamine (DA), in footshock-induced reinstatement of drug seeking¹. In the next sections, each of these

¹ Although not examined in the present dissertation, it is important to note that numerous neurochemical systems other than CRF, NA, and DA, have been shown to play critical role in stress-induced reinstatement. For example, pioneering work of Kalivas and colleagues (e.g., McFarland et al., 2004, Kalivas et al., 2003) has demonstrated that glutamate systems of the PFC, and its release at distal sites (e.g., NAc), mediates footshock-induced reinstatement. Moreover, recent work has implicated glutamate transmission in the VTA in footshock-induced reinstatement of cocaine seeking (Wang et al., 2005, 2007). Results from other recent studies implicate multiple neuropetide systems in stress-induced reinstatement. Most notably, pharmacological manipulation of hypocretin, dynorphin, and neuropeptide Y attenuates footshock-induced reinstatement. This work is the subject of a recent review (see Shalev et al., 2010).

neurochemical systems will be described, and the role that they are known to play in stressinduced reinstatement of drug seeking will be reviewed.

CRF and Stress-Induced Reinstatement

CRF, a 41-residue straight-chain peptide, has been found to play a critical role in the behavioural and neuroendocrine response to stress. The neuropeptide belongs to a family of ligands that includes CRF binding protein, as well as urocortin I, II and III (Bale and Vale, 2004). CRF binds to the 7-domain transmembrane receptors, CRF₁ and CRF₂, which both belong to the class B subtype of G protein-coupled receptors (Chen et al., 1993; Lovenberg et al., 1995). CRF₁ and CRF₂ receptors share approximately 70% amino acid identity, with most of their amino acid differences concentrated in the *N*-terminal ligand binding region, accounting for the substantial differences in the pharmacology of each receptor. Specifically, CRF and urocortin I have a high affinity for CRF₁ receptors, whereas urocortin I, II and III have a high affinity for CRF₂ receptors, with CRF expressing a 10- to 30-fold lower affinity for the CRF₂ receptor than the urocortins (Bale and Vale, 2004).

In its endocrine role, CRF initiates a cascade of events that activates the hypothalamicpituitary-adrenocortical (HPA) axis, also known as the stress axis. Specifically, CRF is released from neurosecretory cells of the paraventricular nucleus (PVN) of the hypothalamus into the median eminence in response to stress. This secretion induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn signals the release of corticosterone from the adrenal cortex into the general circulation (Dunn and Berridge, 1990; Sarnyai et al., 2001).

Independent of its neuroendocrine role, CRF also acts as a neurotransmitter at extrahypothalamic brain sites to mediate various physiological and emotional responses to stress (Sarnyai et al., 2001). Accordingly, CRF cell bodies and receptors are widely distributed throughout the mammalian brain, including regions of the brainstem, limbic forebrain, striatum, and cortex, all of which are importantly involved in the central regulation of the stress response (see Figure 1; Sawchenko et al., 1993; Smagin and Dunn, 2000).

Given the critical role CRF plays in the stress response, it is not surprising that initial explorations of the neurobiology of stress-induced reinstatement of drug seeking focused on this system. Early experiments found that i.e.v. pretreatment with the non-selective CRF receptor antagonists, D-Phe CRF₁₂₋₄₁ and α -helical CRF₉₋₄₁, significantly attenuated or blocked footshock-induced reinstatement of heroin, cocaine, and alcohol seeking (Erb et al., 1998; Le et al., 2000; Liu and Weiss, 2002; Zislis et al., 2007). On the other hand, central injections of CRF, itself, reinstated drug seeking (Shaham et al., 1997; Erb et al., 2006; Mantsch et al., 2008).

The effect of CRF on reinstatement is thought to be mediated by activity at extrahypothalamic brain sites, and independent of its effects on the HPA axis, given that stress-induced reinstatement is largely unaltered by pharmacological or surgical manipulations of the adrenal gland. For example, initial experiments showed that in heroin-trained animals, exposure to footshock following extinction training was equally effective in inducing the reinstatement of drug seeking in adrenalectomized and sham-adrenalectomized rats (Shaham et al., 1997). Similar results were subsequently obtained in cocaine- and alcohol-trained animals (Erb et al., 1998; Le et al., 2000). However, in cocaine-trained animals, a basal level of corticosterone (provided by a corticosterone pellet implanted subcutaneously and corticosterone in the drinking water) was necessary for footshock to induce reinstatement (Erb et al., 1998). Thus, whereas basal corticosterone levels would seem to play a permissive role in footshock-induced reinstatement of cocaine seeking, they do not play this same role in the





Distribution of CRF peptides (top) and receptors (bottom) in the rat brain.

Abbreviations: anterior commissure (ac), nucleus ambiguous (AMB), amygdala (AMYG), anterior olfactory nucleus (AON), area postrema (AP), arcuate nucleus hypothalamus (ARH), Barrington's nucleus (Bar), bed nucleus of the stria terminalis (BST), cerebellum (CBL), corpus callosum (cc), central nucleus of the amygdala (CeA), cochlear nucleus (COCH), caudoputamen (CPU), cerebral cortex (CTX), dorsal column nuclei (D.COL.), deep nucleus of the cerebellum (DEEP.N.), dorsal raphe nucleus (DR), hippocampal formation (HF), inferior colliculus (I.COL.), intermediolateral column of the spinal cord (IML), inferior olivary complex (IO), laterodorsal tegmental nucleus (LDT), lateral geniculate nucleus (LGN), lateral hypothalamic area (LHA), lateral reticular nucleus (LRN), median eminence (ME), medial forebrain bundle (mfb), medial preoptic area (MPO), nucleus of the solitary tract (NTS), olfactory bulb (OB), optic tract (ot), olfactory tubercle (OT), periaqueductal gray (PAG), parabrachial nucleus (PB), pontine gray (PG), perioculomotor raphe (POR), posterior pituitary (PP), pedunculopontine nucleus (PPN), primary sensory nucleus of the trigeminal nerve (PSV), paraventricular nucleus region (PVT), substantia innominata (SI), substantia nigra (SN), supraoptic nucleus (SO), superior olivary complex (SOC), spinal trigeminal nucleus (SpV), stria terminalis (st), supramamillary nucleus (SUM), thalamus (THAL), ventral thalamus (VENT THAL), vestibular nucleus (VEST), ventrolateral medulla (VLM), ventromedial nucleus of hypothalamus (VMH), ventral tegmental area (VTA). Reprinted from Sawchenko et al (2010) Corticotropin-releasing hormone: integration of adaptive responses to stress. In Stress Science: Neuroendocrinology (Fink G, ed), pp355. Oxford, UK: Academic Press. Copyright © Paul E Sawchenko, 2010.

footshock-induced reinstatement of heroin seeking (the importance of basal levels of corticosterone in animals trained to self-administer alcohol is unknown given that in this experiment, all adrenalectomized animals were given corticosterone replacement). The reasons for the discrepancy between heroin and cocaine trained animals in the effects of basal corticosterone levels are unclear, but may be related to the differential effects that repeated exposure of these drugs have on the HPA axis (Erb et al., 1998). For example, there is evidence to suggest that increases in corticosterone and ACTH levels diminish following repeated exposure to opioid agonists (Pechnick, 1993), but remain elevated following repeated injections of cocaine (Torres and Rivier, 1992). One possible consequence of such adaptations is that self-administration of cocaine and heroin becomes differentially state-dependent on the presence of corticosterone. Thus, in the case of cocaine, because cocaine self-administration is accompanied by increases in corticosterone, some minimal level of corticosterone may be required to maintain reinstatement responding. In contrast, heroin self-administration results in the suppression of corticosterone, so adrenalectomy may have minimal consequences on responding during tests for reinstatement (Erb et al., 1998).

Inconsistent with the conclusion that corticosterone does not mediate the footshockinduced reinstatement of cocaine seeking (i.e., Shaham et al., 1997; Erb et al., 1998; Le et al., 2000), Mantsch and Goeders (1999) report that systemic injections of the corticosterone synthesis inhibitor, ketoconazole, significantly attenuate this reinstatement. Importantly, however, ketoconazole acts on several receptor systems in addition to corticosterone, including γ -aminobutyric acid (GABA), histamine and testosterone (Heckman et al., 1992; Gietzen et al., 1996; Fahey et al., 1998), and it is conceivable that its effects on reinstatement are mediated by one or more of these alternate systems. Thus, interpretation of this work should be made with some caution.

The effects of CRF on footshock-induced reinstatement of cocaine seeking have been attributed primarily to its actions at the CRF₁ receptor. For example, administration of the selective CRF₁ receptor antagonist, CP-154,526, prior to footshock stress blocked reinstatement of cocaine and heroin seeking (Shaham et al., 1998). More recently, it has been shown that footshock-induced reinstatement is also blocked by the novel CRF₁ receptor antagonist 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazol[1,2-*b*]pyridazine (MTIP) in animals with a history of alcohol-seeking (Gehlert et al., 2007), and that the highly selective CRF₁ antagonist R278995/CRA0450 attenuates footshock-induced reinstatement in animals with a history of nicotine self-administration (Gehlert et al., 2007). In contrast, pretreatment with the selective CRF₂ receptor antagonist antisauvagine failed to block footshock-induced reinstatement of extinguished nicotine seeking (Gehlert et al., 2007). Similar findings have been reported from studies using the CPP reinstatement paradigm where pretreatment with CP-154,526, but not antisauvagine, blocked footshock-induced reactivation of a morphine CPP (Lu et al., 2000).

Since the original studies demonstrating a critical role for CRF in footshock-induced reinstatement of cocaine seeking, extensive work has been done to localize its effects. This work has revealed a particularly important role for the extended amygdala circuitry, not only for the effects of CRF on footshock-induced reinstatement, but also in the effects of NA on this reinstatement. Next, I will review work establishing a role for NA in footshock-induced reinstatement and subsequently review the localization studies for CRF and NA within the context of a description of the neuroanatomical organization of the extended amygdala circuitry.

Noradrenaline and Stress-induced Reinstatement of Drug Seeking

Like CRF, central NA neurons are also activated by stress and play a key role in the mammalian stress response. All NAergic projections in the brain originate from seven distinctive clusters of NA cell bodies in the brainstem, designated A1-A7 (see Figure 2; Dahlstrom and Fuxe, 1964). These nuclei form two major subgroups: the locus coeruleus (LC), comprised of the A4 and A6 nuclei, and the lateral tegmental (Ltg) nuclei, comprised of the medullary A1-A3 nuclei, and pontine nuclei, A5 and A7. Ascending NAergic projections from these nuclei form two corresponding pathways, the dorsal and ventral pathways, projecting form the LC and Ltg nuclei, respectively. These projections are largely ipsilateral, and collectively innervate the vast majority of the central nervous system (Moore and Bloom, 1979).

The dorsal and ventral NA pathways differ greatly in their overall organization, termination sites, and functional roles. The dorsal pathway is diffuse and provides the sole source of NA input to the cortex, hippocampus, thalamus and cerebellum (Moore and Bloom, 1979). The well-characterized functional role of this system involves different aspects of arousal, cognitive performance, and anxiety (Berridge and Waterhouse, 2003; Sara, 2009). In contrast, the distribution of the ventral pathway is less extensive but, like the dorsal pathway, it provides substantial innervation of subcortical structures, including the hypothalamus and regions of the limbic forebrain, such as the amygdala and bed nucleus of the stria terminalis (BNST; Moore and Bloom, 1979). Of these targets, the amygdala has a uniquely facilitatory influence on the stress response, in contrast to the prefrontal cortex and hippocampus which have been shown to dampen responses to stressful stimuli (Herman and Cullinan, 1997). Together, these innervations contribute to the integration of information about the visceral and physiological status of the organism under conditions of stress.





Distribution of noradrenaline cell body clusters (A1-A7) in the brain stem. *Red*: noadrenaline; *green*: Epinephrine nuclei. Reprinted from Aston-Jones, G (2010) Norepinephrine: CNS pathways and neurophysiology. In Stress Science: Neuroendocrinology (Fink G, ed), pp388. Oxford, UK: Academic Press. Copyright © Gary Aston-Jones, 2010. NA signaling is mediated by five distinct adrenoceptor subtypes, including α_1 , α_2 , β_1 , β_2 and β_3 , all of which are seven transmembrane domain, G protein-coupled receptors (Hieble, 2007). The affinity of NA for α_2 -adrenoceptors exceeds that of α_1 -adrenoceptors, while the affinity of NA for both of these receptors is higher than that for β -adrenoceptors (Nicholas et al., 1996). Neuroanatomical studies show a widespread distribution of all five adrenoceptor subtypes in the brain, except for the β_3 -adrenoceptor, which is mainly expressed in brown adipose tissue (Chamberlain et al., 1999). Both α_1 - and β -adrenoceptors are largely located postsynaptically, whereas α_2 -adrenoceptors are found at both pre- and postsynaptic sites (Nicholas et al., 1996). Presynaptic α_2 -adrenoceptors are the predominant autoreceptor in NA neurons, functioning as the main inhibitory feedback mechanism that controls the release of NA (Gilsbach and Hein, 2008). Thus, administration of α_2 -adrenoceptors antagonists (e.g., yohimbine) at doses known to act at autoreceptors promotes the release of NA, whereas administration of α_2 -adrenoceptors agonists (e.g., clonidine) reduce the release and firing rate of NA neurons (Svensson et al., 1975).

Stress-induced reinstatement studies show that NA, like CRF, plays a critical role in the effects of footshock stress on the reinstatement of drug seeking. For example, systemic injections of α_2 -adrenoceptor agonists, including clonidine, lofexidine and guanabenz, administered at doses known to act at autoreceptors to inhibit NA cell firing and release, block footshock-induced reinstatement of cocaine seeking (Erb et al., 2000). Likewise, central injections of clonidine or its charged analogue, 2-[2,6-diehylphenylamino]-2-imidazole (ST-91), into the lateral or fourth ventricles, block footshock-induced reinstatement of heroin seeking (Shaham et al., 2000b). Consistent with these findings, clonidine has been shown to attenuate footshock-induced reinstatement of nicotine-seeking behaviour (Zislis et al., 2007) and forced swim-induced reinstatement of extinguished cocaine-CPP (Mantsch et al., 2010).

In contrast, systemic injections of the α_2 -adrenoceptor antagonist, yohimbine, reinstates drug-(Lee et al., 2004; Shepard et al., 2004; Le et al., 2005; Feltenstein and See, 2006; Fletcher et al., 2008; Chauvet et al., 2009; Kupferschmidt et al., 2009), and food-seeking (Ghitza et al., 2006; Nair et al., 2009; Nair et al., 2011), and reactivates extinguished cocaine-CPP (Mantsch et al., 2010).

In studies aimed at localizing the effects of NA on stress-induced reinstatement, it was determined that the ventral NA pathway, but not the dorsal pathway, is responsible for the role that this system plays in footshock-induced reinstatement of drug seeking. In one experiment, animals with a prior history of heroin self-administration were given injections of clonidine or ST-91 into the LC, or were given a selective 6-hydroxydopamine (6-OHDA) lesion of the ventral NA pathway. Neither manipulation of the LC affected footshock-induced reinstatement of heroin seeking, whereas selective 6-OHDA lesions of the ventral NA pathway significantly attenuated the effects of footshock on reinstatement (Shaham et al., 2000b). Similarly, selective lesions of the ventral, but not the dorsal, NA pathway inhibited footshock-induced reactivation of morphine-CPP (Wang et al., 2001). Together, these findings are consistent with a larger body of work indicating an important role for the ventral NA pathway in other drug-related behaviors, including the aversive and anxiogenic effects of cocaine and heroin withdrawal (Aston-Jones et al., 1999; Shaham et al., 2000b; Wang et al., 2001).

Localization of CRF and NA Effects on Footshock-induced Reinstatement of Drug Seeking

The effects of both CRF and NA on reinstatement of drug seeking have been localized to the BNST and central nucleus of the amygdala (CeA), primary components of the extended amygdala that have been implicated in fear, anxiety, and other stress-related responses (Everitt et al., 1989; Numan, 1996; Davis et al., 1997; Holahan et al., 1997). The extended amygdala circuitry comprises a continuum of neurons extending from the CeA, through the substantia inominata and BNST, to the shell of the nucleus accumbens (NAc; Alheid, 2003). This conceptualization of the extended amygdala was proposed by de Olmos and colleagues (de Olmos and Heimer, 1999; Alheid, 2003) based on neuroanatomical studies demonstrating an extensive system of intrinsic interconnections amongst regions of the basal forebrain, including the BNST and CeA, and considerable similarities in the distributions and neurochemical phenotypes of projections to and from these regions.

Within both the BNST and CeA, CRF-immunoreactive neurons and CRF mRNA are found primarily in the lateral division of both structures (Swanson et al., 1983; Sakanaka et al., 1986; Potter et al., 1994). These neurons project to various hypothalamic, brainstem and midbrain regions, including the ventral tegmental area (VTA), as well as between the CeA and BNST (Sakanaka et al., 1986; Rodaros et al., 2007). Notably, the CeA provides an important source of CRF to the BNST (Sakanaka et al., 1986), and the lateral BNST itself contains intrinsic CRF projections from, primarily, dorsal to ventral subregions (Phelix and Paull, 1990; Veinante et al., 1997). As shown in Figure 1, both CRF₁ and CRF₂ receptors are expressed in the BNST, however only CRF₁ receptors are expressed in the CeA (Chalmers et al., 1995; Van Pett et al., 2000).

Enzymatic-isotopic assay for NA content within discrete brain regions of the extended amygdala reveals that the highest NAergic content in the brain occurs in the ventral BNST, with moderately high content found in the dorsal BNST and CeA (Brownstein et al., 1974; Versteeg et al., 1976). Retrograde-labelling experiments have identified these NAergic fibers as originating primarily in A1/A2 medullary nuclei (Aston-Jones et al., 1999). Neuroanatomical studies have shown no clear overall association between any particular

adrenoceptor subtype and the terminal regions of either the ventral or dorsal NA pathway (Nicholas et al., 1996). Indeed, both β_1 - and β_2 –adrenoceptors are quite evenly expressed in the CeA and BNST (Rainbow et al., 1984). In addition, the CeA has been shown to have high expression of both α_1 - and α_2 -adrenoceptors, whereas the BNST shows only moderate expression of α_1 - but intense expression of α_2 -adrenoceptors (Talley et al., 1996; Day et al., 1997).

In a series of experiments using local pharmacological manipulations, Stewart and colleagues (Erb and Stewart, 1999; Leri et al., 2002) localized the effects of NA and CRF on footshock-induced reinstatement to the BNST and CeA. In one such study, Erb and Stewart (1999) administered bilateral injections of the non-selective CRF receptor antagonist, D-Phe, into the BNST and CeA. Antagonism of CRF receptors within the BNST, but not the CeA, blocked the 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. Together, these findings demonstrate a critical role for the activation of CRF receptors in the BNST, but not CeA, in the mediation of stress-induced reinstatement (Erb and Stewart, 1999). Consistent with these findings, Wang et al (2006) reported that local injections of the CRF₁ antagonist, CP-154,526, into the BNST, but not the amygdala, attenuated footshock-induced reinstatement of morphine CPP.

Although both studies indicate that CRF receptors in the CeA do not alter the effects of footshock on the reinstatement of cocaine seeking, it is important to note that neuroanatomical studies reveal a substantial CRF projection from the CeA to the BNST (Sakanaka et al., 1986). Erb and colleagues (2001) demonstrated that a functional disconnection of this CRF pathway, using an asymmetric lesion procedure, attenuated the effects of footshock stress on the reinstatement of cocaine seeking. Thus, although activation of CRF receptors in the CeA is not

critical for stress-induced reinstatement of cocaine seeking, the CeA provides an important source of CRF in the BNST in mediating reinstatement (Erb et al., 2001).

In a study designed to localize the effects of NA on footshock-induced reinstatement, Leri et al (2002) administered local injections of the $\beta_{1/2}$ -adrenoceptor antagonists, betaxolol and ICI-118,551, bilaterally into the CeA and BNST. Blockade of $\beta_{1/2}$ -adrenoceptors in the BNST dose-dependently attenuated footshock-induced reinstatement, whereas a complete blockade of stress-induced reinstatement was observed at all doses following injection into CeA. Taken together, these data suggest that footshock-induced release of NA in the BNST and CeA is critical to the reinstatement of cocaine seeking.

In addition to the similar role NA and CRF systems play in stress-induced reinstatement, there is also considerable evidence of synaptic convergence between the two systems within the CeA and BNST. For example, a distinct population of CRFimmunoreactive neurons in the ventromedial BNST surrounded by DA β -hydroxylase (DBH)immunreactive fibers, which have been demonstrated through double-label immunocytochemistry to synapse directly onto CRF-containing perikarya, has been identified (Hornby and Piekut, 1989; Phelix et al., 1994). In the CeA, CRF-immunoreactive neurons have been shown to occur in close proximity to NA terminals, but not as dense as that seen in the BNST (Hornby and Piekut, 1989). More recently, it has been shown that β_1 -adrenoceptors are expressed on CRF-ir neurons in the CeA (Rudoy et al., 2009). In this same study it was shown that administration of the β_1 -adrenoceptor antagonist, betaxolol, during the early cocaine withdrawal period significantly attenuated increases in CRF mRNA expression in the CeA. Taken together, these findings suggest NAergic signaling through the β_1 -adrenoceptor modulates CRF gene expression (Rudoy et al., 2009). Based on the important roles played by CRF and NA in the effects of stress on reinstatement, and compelling anatomical evidence for their overlap in the CeA and BNST, it is plausible that an interaction between these systems contributes to the reinstatement of drug seeking. To date, however, there are no data to confirm such an interaction, much less the direction of the interaction, should one exist. A major focus of the present dissertation is to provide a direct exploration of this interaction (see Thesis Objectives section).

DA and Stress-induced Reinstatement

It is well known that exposure to aversive stimuli, such as footshock, increases DA release in terminal regions of the mesocorticolimbic DA circuitry (Abercrombie et al., 1989; Gresch et al., 1994; Kalivas and Duffy, 1995). Originating within the VTA (A10), the mesocoritcolimbic DA system innervates virtually every nucleus in the limbic system including the NAc, medial prefrontal cortex (mPFC), amygdala, hippocampus, and ventral pallidum (Swanson, 1982; Oades and Halliday, 1987). DA signaling is mediated by five major receptor subtypes, designated D₁-D₅, all of which belong to the seven transmembrane domain G protein-coupled family of receptors. Each DA receptor subtype is commonly categorized as either D₁-like (D₁ and D₅) or D₂-like (D₂, D₃ and D₄) based on sequence, homology and pharmacology (Jackson and Westlind-Danielsson, 1994). D₁-like receptors are largely located postsynaptically, whereas D₂-like receptors are located both pre- and postsynaptically (Levey et al., 1993). Like the α_2 -adrenoceptor of the NAergic system, presynaptic D₂-like receptors act as autoreceptors, slowing the firing rate of DA neurons as well as inhibiting DA synthesis and release (Goldstein et al., 1990). The expression, distribution and density of D₁- versus D₂like receptors in terminal regions of the VTA DA projection varies, however, D₁-like receptors are usually present in higher density than D₂-like receptors (Boyson et al., 1986).

Although DA is perhaps most commonly associated with the positive reinforcing effects of drug and non-drug reinforcers, it has been found to play an important role in the neurobiology of fear and anxiety (Pezze and Feldon, 2004; de Oliveira et al., 2006; Macedo et al., 2007) and has been indirectly implicated in some of the anxiogenic and anhedonic effects of cocaine withdrawal (Ackerman and White, 1992; Koob, 1992). There is also recent evidence implicating DA in the footshock-induced reinstatement of cocaine seeking. For example, in a series of experiments Capriles and colleagues (2003) examined whether activity at D₁- and/or D₂-like receptors in the mPFC and orbitofrontal cortex (OFC) mediated footshock-induced reinstatement of cocaine seeking, by injecting selective DA antagonists into these areas. The results showed that local administrations of the D₁-like, but not D₂-like, antagonists blocked footshock-induced reinstatement (Capriles et al., 2003). Consistent with these findings, subsequent experiments have shown that administration of the mixed D₁/D₂-like receptor antagonist fluphenazine into the dorsal PFC blocks footshock-induced reinstatement, as does temporary inactivation of the VTA (McFarland et al., 2004).

A second major terminal region of the VTA DA projection is the NAc, which, like the PFC, shows enhanced DA levels in response to stress (Sorg and Kalivas, 1991; Kalivas and Duffy, 1995). In a series of experiments, McFarland and colleagues (2004) examined the role of the NAc in footshock-induced reinstatement of cocaine seeking, and showed that temporary inactivation of both the NAc shell and core blocks footshock-induced reinstatement. Local infusion of the D_1/D_2 -like receptor antagonist fluphenazine into the NAc core failed to block footshock-induced reinstatement, suggesting that activity at DA receptors in this region does not mediate this behaviour. Consistent with these findings, the authors also show that exposure to footshock increases glutamate, but not dopamine, levels in the NAc core, which is blocked by inhibition of the dorsal PFC. Taken together, these data suggest that footshock-induced

reinstatement depends on the activation of VTA DA neurons projecting to the PFC, which in turn stimulates glutamate release in the NAc (McFarland et al., 2004).

Interaction Between the Mesocortical DA System and CRF in Stress-induced Reinstatement

It has recently been suggested that CRF may activate the mesocortical DA system to induce reinstatement by footshock stress. This proposed interaction between CRF and DA systems is based on several lines of behavioural and neuroanatomical evidence, which together demonstrate a functional convergence between these systems in brain regions known to play an important role in stress-induced reinstatement. For example, central administration of CRF induces the release of DA in the PFC (Lavicky and Dunn, 1993), and increases the firing rate of VTA DA neurons in a dose-dependent manner (Wanat et al., 2008). In addition, exposure to footshock stress induces the release of CRF in the VTA, which results in a significant increase of local (and presumably distal) DA levels in the VTA that is blocked by local administration of the CRF receptor antagonist, α -helical CRF (Wang et al., 2005). Consistent with these findings, Ungless and colleagues (2003) showed that application of CRF to midbrain DA neurons induces a potentiation of NMDA receptor-mediated excitatory postsynaptic potentials. Finally, neuroanatomical studies have shown that the VTA expresses CRF₁ receptors, although in lower levels than other brain regions (Chalmers et al., 1995; Van Pett et al., 2000), and receives CRF inputs from the CeA, BNST and PVN (Swanson and Sawchenko, 1983; Rodaros et al., 2007).

Recent studies have revealed that most synapses between CRF-containing neurons and DA neurons in the VTA are asymmetric and glutamatergic, suggesting that glutamate releasing neurons are also part of the neuronal circuitry mediating the effects of CRF in VTA DA
neurons (Tagliaferro and Morales, 2008). Consistent with this idea, a series of microdialysis studies examining the role of CRF, DA and glutamate release in the VTA following exposure in footshock-induced reinstatement showed that in cocaine-experienced, but not drug naïve animals, CRF gains control over local glutamate release. Specifically, footshock-induced release of DA and glutamate release in the VTA, as well as footshock-induced reinstatement of cocaine seeking, were blocked by intra-VTA infusion of the CRF antagonist α -helical CRF (Wang et al., 2005).

Subsequent experiments designed to explore the role of CRF receptor subtypes involved in footshock-induced release of DA and glutamate in the VTA, have shown that CRF₂ but not CRF₁, receptors are involved in this effect. Specifically, intra-VTA infusion of the CRF₂ antagonist, antisauvagine, blocks footshock-induced reinstatement and the associated increase of DA and glutamate. In contrast, intra-VTA infusion of the selective CRF₁ antagonists, NBI-27914 and R121919, failed to block footshock-induced reinstatement and local increases in DA and glutamate levels (Wang et al., 2007). These findings are consistent with electrophysiological studies showing that CRF-induced potentiation of N-methyl-Daspartate (NMDA) receptor-mediated transmission in VTA DA neurons is blocked with pretreatment of antisauvagine, but not the CRF₁ receptor antagonist CP-154,526 (Ungless et al., 2003). Together, these results suggest that activity at VTA CRF₂ receptors plays an important role in the behavioural and neurophysiological response to stress.

Taken together, the behavioural and neuroanatomical evidence indicate that an interaction between CRF and DA systems, at the level of the VTA, likely contributes to the reinstatement of cocaine seeking by footshock. However, it is currently unknown whether DA systems mediate CRF-induced reinstatement, and should DA mechanisms be involved, it is also unknown which DA receptor subtype mediates the effect.

Neurocircuitry Mediating Stress-induced Reinstatement

Based on the substantial neuroanatomical and behavioural evidence reviewed here, a model of the neurocircuitry mediating stress-induced reinstatement can be described (Figure 3). This model, which has been described in several iterations (e.g., Shaham et al., 2003; Stewart, 2003; Bossert et al., 2005; Wise and Morales, 2010), proposes that following extinction of drug-seeking behaviour, exposure to footshock stress activates NA neurons of the Ltg nuclei (green), which leads to the downstream release of NA in regions of the CeA and BNST that express CRF neurons (blue). Based on the neuroanatomical evidence showing that β_1 -adrenoceptors are expressed on CRF neurons in the CeA (Rudoy et al., 2009), and that NA terminals make synaptic contact with CRF neurons in the BNST (Phelix et al., 1994), it can be further proposed that NA and CRF systems interact within these brain regions to mediate stress-induced reinstatement. More specifically, it can be hypothesized that within the CeA, NA binds postsynaptically to $\beta_{1/2}$ -adrenergic receptors located on CRF neurons (green diamonds), activating both CRF neurons projecting to the BNST, as well as CRF neurons projecting to distal structures, including the VTA. Likewise, within the BNST NA binds postsynaptically to $\beta_{1/2}$ -adrenoceptors receptors located on CRF neurons (green diamonds), stimulating local CRF release within the BNST, as well as distal CRF release in the VTA.

Based on the work of Wang and colleagues (2005, 2007), the downstream release of CRF into the VTA facilitates DA and glutamate transmission in the region to, in turn, facilitate reinstatement of cocaine seeking. Thus, it can be further hypothesized that the sources of CRF in the VTA following exposure to footshock are the CeA and BNST (Rodaros et al., 2007), and that CRF-induced signaling in the VTA ultimately causes downstream release of DA in the

Figure 3



Diagram depicting the neurochemical pathways and brain regions implicated in stress-induced reinstatement.

mPFC (orange). Finally, activation of D₁-like receptors in the mPFC (Capriles et al., 2003; McFarland et al., 2004) in turn activate a glutamatergic projection to the NAcc core (purple; McFarland et al 2004), as a final output pathway mediating footshock-induced reinstatement.

Thesis Objectives

The series of experiments presented in this dissertation constitute a further exploration of the stress-related neurochemical circuits mediating the reinstatement of cocaine seeking. Specifically, the experiments were designed to explore whether central CRF and catecholamine (NA and DA) systems functionally interact to mediate the reinstatement of cocaine seeking in rats. To this end, I developed a series of three research objectives based on what is currently known about the role of CRF, NA and DA systems in stress-induced reinstatement to test a series of hypotheses consistent with the neuroanatomical model just described. The first objective, which is the focus of Chapter 3 experiments, sought to determine whether NA and CRF systems functionally interact to mediate reinstatement of cocaine seeking and, if so, to determine the direction of this interaction. The second objective, which is explored in experiments outlined in Chapter 4, was to consider possible interactions between central CRF and DA systems, by determining whether selective antagonism of DA receptors interferes in CRF-induced reinstatement of cocaine seeking.

The third and final objective, which is addressed in Chapter 5, was based largely on an unexpected outcome of an experiment presented in Chapter 3. Specifically, in Experiment 4 (Chapter 3), antagonism of CRF receptors failed to interfere in the effects of the α_2 -adrenoceptor antagonist, yohimbine, on the reinstatement of cocaine seeking. These findings contradicted my hypothesis that antagonism of CRF receptors would block the downstream effects of yohimbine-induced NA transmission and, thereby, interfere in yohimbine-induced

reinstatement of cocaine seeking (a detailed rationale for this hypothesis can be found in the introduction of Chapter 3). It is important to note, however, that the pharmacological profile of yohimbine also includes a significant affinity for receptors other than the α_2 -adrenoceptor, including DA and serotonin (5-HT)_{1A} receptors (Millan et al., 2000), and that CRF has also been shown to interact with these systems (Rodaros et al., 2007; Dzung Le et al., 2009). Thus, it is possible that yohimbine may act through neurochemical systems other than, or in addition to, NA to mediate reinstatement. The objective of this chapter was to explore the roles of NA and DA in yohimbine-induced reinstatement of cocaine seeking. To this end, I determined whether selective antagonism of the α_2 -adrenoceptor as well as D₁- and D₂-like receptors would interfere in yohimbine-induced reinstatement of cocaine seeking.

The research objectives upon which the present work was based were achieved by combining several methods, including behavioural (reinstatement procedure), pharmacological (systemic and central injections of selective agonists and antagonists), and molecular (*in situ* hybridization). All relevant procedural details for each methodology are described in the General Methods section (Chapter 2). In addition, detailed descriptions of the specific parameters utilized in each study are outlined in the Methods section of each experiment (Chapters 3-5).

Rationale for Studies in Cocaine-trained Rats

All experiments described in this dissertation were carried out in cocaine-trained animals. Cocaine is a highly reinforcing psychostimulant in both humans and animals. The primary neuropharmacological effect of cocaine is the blockade of neuronal reuptake mechanisms for the monoamine neurotransmitters NA, DA and 5-HT at the presynaptic junction (Reith et al., 1986). Consequently, the concentration of monamines in the synaptic cleft is increased, resulting in higher levels of postsynaptic activation.

I focused on cocaine in this work in part because it is considered one of the major drugs of abuse in modern society, and represents the third most widely used illicit drug in Canada, following cannabis and hallucinogens (Adlaf et al., 2005). Despite the prevalence of cocaine dependence, however, little progress has been made in the development of successful treatment and prevention programs. Indeed, most individuals who achieve sustained abstinence do so only after a number of cycles of detoxification and relapse (Sinha et al., 1999; Sinha, 2009). Thus, it is critical that studies be carried out at a basic research level to further our understanding of the behavioural and neurobiological effects of cocaine abuse and the phenomenology of relapse. The present experiments, which are part of a larger ongoing research program in my laboratory, are designed to contribute to this body of work. CHAPTER 2

General Methods

Chapter 2: General Methods

Animals

Male Long-Evans rats (275-300g; Charles River, Canada) were used in all experiments. Animals were individually housed in a temperature- $(21\pm1^{\circ}C)$ and humidity-controlled vivarium, and maintained on a 12-h light-dark cycle (lights on 1900-0700 h). Standard rat chow and water were freely available. At least 1 week of acclimatization to the vivarium was given prior to surgery, and an additional week of recovery was allowed between surgery and the onset of behavioural testing. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee.

Surgery

Intravenous (i.v.) Catheterization

Under gas anesthesia (3-5% isoflurane; Benson Medical, Markham, ON, Canada), animals in drug self-administration studies were surgically implanted with an intravenous (i.v.) silastic catheter (Dow Corning; 3-cm length, inner diameter 0.5mm, outer diameter 0.9mm) into the right jugular vein. The i.v. catheter was secured to the vein with silk sutures. The catheter was then passed subcutaneously to the top of the skull where it exited into a modified cannula (22 gauge; Plastics One, Roanoke, VA, USA), which was mounted to the skull with jeweler's screws and dental cement. A plastic blocker was placed over the open end of the cannula for the duration of the recovery period, to maintain catheter patency.

Before the start of self-administration training, animals were given a minimum 7-day recovery period. During self-administration training, catheters were flushed following each

session with 0.2 ml of a 50-50% heparin (1,000 i.u./ml; Leo Pharma Inc., Thornhill, ON, Canada)-dextrose (Hospira, Montreal, QC, Canada) solution, to maintain catheter patency.

Intracerebroventricular (i.c.v.) Cannulation

During i.v. surgery, rats from Experiments 1-7 were also implanted with a 22-gauge guide cannula (18 mm; Plastics One) into the left lateral ventricle, according to the following stereotaxic coordinates: AP -1.0 mm, ML +1.4 mm, and DV -3.7 mm (Paxinos and Watson, 1998). These coordinates positioned the cannula 1 mm above the ventricle. A stainless steel dummy (Plastics One), extending 1 mm beyond the tip of the cannula to the injection site, was inserted after surgery. Cannula placements were verified by observation of drinking behaviour following i.c.v. administration of angiotensin (50 μ g/2 μ l, i.c.v.; 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 2-3 min (Sakai et al., 1995).

Microinjection Procedures

I.c.v. injections of all substances (CRF, D-Phe CRF₁₂₋₄₁, NA bitartrate) were given in a volume of 4 μ l, using a 10 μ l Hamilton syringe connected to a 30-gauge injector (Plastics One). Injections were made over 2 min, and the injectors were left in place for an additional 1 min.

Drugs

Cocaine HCl was obtained from Medisca Pharmaceuticals (St. Laurent, QC, Canada) and dissolved in sterile, physiological saline. Pharmacological compounds used to manipulate CRF systems, including human/rat CRF (Sigma-Aldrich, Oakville, ON, Canada) and the CRF receptor antagonist D-Phe CRF₁₂₋₄₁ (Bachem, Torrance, CA, USA), were dissolved in physiological saline. Pharmacological compounds used to activate NAergic systems, including the α_2 -adrenoceptor antagonist yohimbine and NA bitartrate (both obtained from Sigma-Aldrich) were dissolved in distilled water (pH 6.7) and physiological saline, respectively. The α_2 -adrenoceptor agonist clonidine (Sigma-Aldrich), administered at doses used to block NAergic cell firing and release, was dissolved in physiological saline. DA antagonists, including the D₁-like receptor antagonists SCH23390 (Sigma-Aldrich) and SCH31966 (Tocris Bioscience, Ellisville, MO, USA), as well as the D₂-like receptor antagonist raclopride (Sigma-Aldrich), were all dissolved in physiological saline.

Apparatus

Drug Self-administration Chambers

Drug self-administration chambers were equipped with two retractable levers (Med Associates, St Albans, VT, USA) located 9 cm above the floor. An infusion pump (Razel Scientific Instruments, Stamford, CN, USA) was activated by responses on one lever ("active lever"), resulting in the delivery of cocaine solution over a 3-sec period in a volume of 65 µl. Each active lever response was automatically recorded using a computer interface and software (Med Associates). Responses on the second lever ("inactive lever") were also 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.

Sucrose Pellet Self-administration Chambers

Similar to the drug self-administration chambers, sucrose pellet self-administration chambers were also equipped with two retractable levers (Med Associates) located 9 cm above

the floor, with a 3 cm x 3 cm trough pellet receptacle located in between them. The pellet receptacle was also located 9 cm above the floor. A modular pellet dispenser (Med Associates) was activated by responses on one lever ("active lever"), resulting in the release of one sucrose pellet into the pellet receptacle. Each active lever response was automatically recorded using a computer interface and software (Med Associates). Responses on the second lever ("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.

Procedures

Reinstatement of Drug Seeking

As shown in Figure 4, all reinstatement experiments were carried out in four phases: (*1*) self-administration training (8-10 days); (*2*) drug-free period (7 days); (*3*) extinction training (3 days); and (*4*) testing for reinstatement (2 days).

Phase 1: Self-administration Training

Prior to self-administration training, rats were habituated to the self-administration chambers for a 2-h session, during which time the active lever remained retracted, and the inactive lever remained extended. Twenty-four to 48 hours following habituation, rats were divided into 2 groups (squad 1 and 2) and trained to self-administer cocaine (0.23 mg/65 µl infusion, i.v.) on a fixed-ratio-1 (FR-1) schedule of reinforcement, during daily 180-min morning (3–4 h after lights off) or afternoon (6–7 h after lights off) sessions. On the first training day, animals received a single priming infusion of cocaine (in a volume of approximately 65 µl) at the beginning of the session in order to fill the catheter with the drug.

Figure 4



Diagram depicting the general timeline of the stress-induced reinstatement procedures (A). Training: animals are given daily, 3-h self-administration sessions for 8-10 days followed by a 7-day drug free period; *Extinction (B)*: animals are given four 60-min extinction sessions (lever extended), each separated by a 30-min period of active lever retraction (lever retraction) for 3 consecutive days. Extinction Day 3 was modified to include sham i.p. and/or i.c.v. injections to acclimatize animals to test procedures; *Tests For Reinstatement (C)*: animals were given 2-3 tests for reinstatement 24-h apart. Test days began with extinction sessions until animals reached criterion (15 or fewer responses on the "active" lever). Once the criterion was reached, animals administered the test substance (NA, CRF or YOH). In some experiments, animals were pretreated with a selective agonist or antagonist prior to administration of the test substance. Each day squad 1 and 2 alternated between self-administering during a morning or afternoon training session. 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. Training conditions were implemented for 8-10 training 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. Subsequently, the availability of cocaine 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. During the self-administration session, responses on the active lever resulted in a 3-s infusion of cocaine (in a volume of 65 μ l) and 20-s illumination of the stimulus light which represented a "time-out" period, during which time responses were recorded but did not lead to further reinforcement. Responses on the inactive lever were recorded throughout the 180-min session, but were without consequence.

Phase 2: Drug-free Period

Immediately following the last day of training, rats were kept in their homecages and maintained under the vivarium conditions (see Animals section above), for a 7-day drug-free period. A drug-free period of 7 days was selected to ensure that testing for reinstatement occurred at a time that exceeded the initial cocaine-withdrawal period (typically defined as the first 24-48 h following cessation of drug exposure), given that the objective of these studies was to examine the effects of stress on the long-term reinstatement of drug seeking.

Phase 3: Extinction Training

In all experiments, animals were given 3 consecutive days of extinction training following the drug-free period. During extinction, activation parameters of the self-administration equipment (houselight light, stimulus light and infusion pump) remained the same as those during self-administration training, only responses on the active lever did not result in an infusion of drug.

On Extinction Days 1 and 2, each session began with the active lever retracted and inactive lever extended for 30 min. Following this time period, the active lever was extended for 60 min. This cycle of 30-min active lever retraction and 60-min extension was repeated a total of 4 times in each day (see Figure 4B). On Day 3 of extinction, the cycle of active lever retraction and extension were the same as those employed on Day 1 and 2, except animals were given sham intarperitoneal (i.p.) and/or sham i.c.v. injections after the first extinction session to acclimatize animals to the testing manipulations that occurred in the subsequent reinstatement test phase (specific parameters are described in each experiments).

Phase 4: Testing for Reinstatement

Following extinction, animals were given 2-3 tests for reinstatement 24 hours apart (see Figure 4C). During tests for reinstatement, activation parameters for the self-administration equipment (houselight light, stimulus light and infusion pump) remained the same as those for extinction training, in that lever pressing was without consequence. Based on the cocaine self-administration and extinction data, animals were divided into test conditions such that there were no significant differences in cocaine intake or number of lever responses during the final extinction session (i.e., session 4, Extinction Day 3).

Test days began with extinction conditions identical to those used during the extinction phase (30-min active lever retraction and 60-min active lever extension; see Figure 4C) until the number of responses on the active lever was 15 or fewer. If during the first extinction session the number of responses exceeded 15, additional extinction session were implemented until this criterion was reached. Once this criterion for responding was reached, animals were administered the test substance (NA [Exp 1A, 2], CRF [Exp 3, 5, 6, 7] or yohimbine [Exp 4, 10-12, 14]) or its vehicle. In all experiments, each animal was tested with both the test substance and the vehicle on alternate days, in a counterbalanced order.

In some experiments, animals were pretreated with a selective agonist or antagonist prior to the administration of the test substance (Exp 2-7, 11, 12, 14). In these experiments, different groups of animals were administered different doses of the pretreatment compound (justifications for doses selected are described in Drug section of each experiment). At an appropriate time after administration of the test substance or its vehicle (5-30 min), determined on the basis of the pharmacological profile for the individual substance (see individual experiments), the previously drug-reinforced lever was extended and a 60- to 180-min test for reinstatement was conducted.

Self-administration of Sucrose Pellets

In Chapters 4 and 5 (Exp 8, 9 and 13), animals were trained to self-administer sucrose pellets in order to test for non-specific motoric effects of D_1 -like receptor antagonists, SCH23390 and SCH31966, and the D_2 -like receptor antagonist, raclopride, on lever pressing behaviour. A timeline of the procedures used can be seen in Figure 5.

For 2-3 consecutive days prior to sucrose self-administration, animals were given 10-15 sucrose pellets (per day) in the home cage to familiarize them to the food. Animals

Figure 5

Self-Administration (8 Days)		Acclimatization (1 Day)		Non-Specific Motoric Testing (3-5 Days)						
		Sham IP		Test Substance			Test Substance			
_	Training	↓	Training	↓	Test Day 1		Maintenance	↓		Test Day 2
LR	LE	LR	LE	LR	LE	// LR	LE	//	LR	LE
5	180 min	5	180 min	30	180	// 5	180	//	30	180 min

Diagram depicting the general timeline of sucrose pellet self-administration procedures. *Training*: animals are given daily, 3-h self-administration sessions for a minimum of 8 days; *Acclimatization:* once stable responding (<10% variability) was achieved, animals received a sham i.p. injection to acclimatize animals to test procedures; *Tests For Non-Specific Motoric Effects:* animals were given 2-3 tests, each 48-h apart. Test days were separated with intervening maintenance sucrose pellet self-administration sessions.

were initially habituated to the sucrose self-administration chambers for a 2-h session, approximately 24- to 48-h before the first training session. During habituation, the active lever remained retracted and the inactive lever remained extended. Animals were divided into 2 groups (squad 1 and 2) and trained to lever press for sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ, USA) on a FR-1 schedule of reinforcement for a total of 8-10 days. Squad 1 animals were always trained in morning (3–4 h after lights off) sessions and squad 2 animals were always trained in afternoon (6–7 h after lights off) sessions. Subsequent tests for nonspecific motoric effects of the various DA antagonists were given at a time of day that corresponded to the animals' training session. Animals were not food deprived at any point during sucrose self-administration experiments.

Similar to those parameters described for the self-administration of cocaine (see Phase 1 of the Reinstatement procedure), each sucrose pellet training session began with a 5-min acclimatization period, during which time 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. During the sucrose self-administration session, 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 that represented a "time-out" period, during which time responses were recorded but did not lead to further sucrose reinforcement. Responses on the inactive lever were recorded throughout the 180-min session, but were without consequence.

Once animals exhibited a stable number of lever responses (<10% variability) in 2 consecutive sessions, following a minimum of 8 sucrose training sessions, a sham i.p. injection was given prior to the next training session to acclimatize animals to manipulations that

occurred in the subsequent test phase. Following the sham injection, animals were placed in the chamber and 30-min later, the final training session began. This 30-min period coincided with the time given after administration of the DA antagonist or its vehicle (see Figure 5).

For 2-3 sessions immediately following the final training session, tests for non-specific motoric effects of the test substances began. During the test phase, different groups of animals were pretreated with either one of the D₁-like receptor antagonists, SCH23390 (0, 0.05, 0.1 mg/kg) or SCH31966 (0, 0.2 mg/kg), or the D₂-like receptor antagonist, raclopride (0, 0.25, 0.5 mg/kg), 30 min prior to the test session. For each antagonist, every animal was tested under vehicle and two dose conditions (see individual experiments for details). Test sessions were separated by 48 h, with intervening baseline sucrose pellet self-administration sessions.

In Situ Hybridization and Autoradiography

For *in situ* hybridization studies (see Chapter 3, Experiment 1B), rats were exposed briefly (30-45 s) to isoflurane vapors and then killed by decapitation. The brains were rapidly removed, flash-frozen in isopentane (-35 to -40°C) and stored at -86°C until processing for *in situ* hybridization.

Coronal brain sections (12 µm) were collected at -20°C through all levels of the BNST, CeA, and PVN. Sections were thaw mounted onto glass slides (Fisher Scientific) and stored at -86°C. Subsequently, slide-mounted frozen sections were thawed and then pre-hybridized at room temperature. The sections were fixed in 4% paraformaldehyde for 5 min, then rinsed in 1X PBS (2X 5 min). The sections were treated with 0.1M triethanolamine-HCl for 5 min, then acetylated in 0.1M triethanolamine-HCl containing 0.25% acetic anhydride for 10 min, followed by two 5-min rinses in 2X SSC. The slides were then dehydrated in graded ethanol, defatted in 100% chloroform for 5 min and hydrated. Hybridization for c-fos mRNA was

performed using ³⁵S-UTP labeled riboprobes complementary to bases 479–498 (5'gggagtggtgaagaccatgt-3') and bases 961–942 (5'-ctgaaggctgaaccctttga-3'; GenBank no. NM_022197.2). Hybridization for CRF mRNA was performed using ³⁵S-UTP labeled riboprobes complementary to bases 690–709 (5'-gctcagcaagctcacagcaa-3') and bases 1066– 1047 (5'-agcagatggaagtcacccagttgtt-3'; GenBank no. NM_031019.1). The probe was diluted to 18,000 cpm/µL in hybridization solution containing 50% formamide, 35% Denhardt's solution, 10% dextran sulfate, 0.1X standard sodium citrate (SSC), salmon sperm DNA (300 µg/mL), yeast tRNA (100 µg/mL), and dithiothreitol (40 µM) and incubated overnight at 60°C. Slides were rinsed with agitation using decreasing concentrations of SSC, dipped in Milli-Q water, dehydrated in 70% ethanol, and air-dried. The slides were exposed to Kodak BioMax film at 4°C for 7 days for c-fos mRNA and 2-7 days for CRF mRNA.

Autoradiographs of brain sections were used to quantify mRNA levels with computerassisted image analysis (MCID, InterFocus, Inc., Leiton, UK). Standard curves obtained from calibrated radioactive standards were used to convert film optical densities to micro-Curie per gram. The brain regions examined were identified using the Paxinos and Watson (1998) atlas. Each region was sampled under uniform background illumination conditions in 3-4 tissue sections. Densitometry data for each region were averaged across brain sections for a given subject, then for all subjects within a given treatment group.

CHAPTER 3

Interactions between NA and CRF in the Reinstatement of

Cocaine Seeking

Chapter 3: Interactions between NA and CRF in the Reinstatement of

Cocaine Seeking

The primary objective of the experiments presented in this chapter was to determine whether NA and CRF systems interact functionally to mediate the reinstatement of cocaine seeking and, should an interaction exist, to determine the direction of that interaction. A series of five experiments was conducted to address these research questions.

Experiment 1 was carried out to establish a method for inducing reinstatement of cocaine seeking via selective activation of NA receptors, and to determine whether this method would correspond to up-regulation of genetic markers of neuronal and neurochemical activation within brain regions known to mediate the effects of stress on reinstatement. Several different pharmacological agents that act to principally activate NA systems have been utilized in reinstatement studies to investigate the role NA plays in this behaviour. The most commonly used agents include the α_2 -adrenoceptor antagonists (e.g., yohimbine, RS79948) and selective NA reuptake inhibitors (e.g., nisoxetine). Other agents less frequently used include α_1 –adrenoceptor agonists (e.g., prazosin) and selective $\beta_{1,2}$ -adrenoceptor agonists (e.g., carazolol, salbutamol).

Both RS79948 and nisoxetine have potent and selective affinity for their respective target sights. Specifically, RS79948 has a high affinity for the α_{2A} - α_{2B} - and α_{2C} -adrenoceptor (Uhlen et al., 1998). Similarly, nisoxetine potently blocks the high affinity uptake of [³H]NA in the rat brain by binding to presynaptic uptake sites for NA (Wong et al., 1982; Tejani-Butt et al., 1990). However, both RS79948 and nisoxetine fail to induce the reinstatement of extinguished drug (Le et al., 2005; Schmidt and Pierce, 2006) or food (Nair et al., 2010) seeking. In contrast, yohimbine has been shown to reliably induce the reinstatement of extinguished cocaine (Lee et al., 2004; Shepard et al., 2004; Feltenstein and See, 2006;

Fletcher et al., 2008; Chauvet et al., 2009; Kupferschmidt et al., 2009), alcohol (Le et al., 2005) and food (Ghitza et al., 2006; Nair et al., 2009; Nair et al., 2011) seeking in rats, despite having less selectivity for the α_{2A} - α_{2B} - and α_{2C} -adrenoceptors than RS79948. In addition, yohimbine also has a significant affinity to receptors other than the α_2 -adrenoceptor, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and D₂-like receptors (Scatton et al., 1980; Pettibone et al., 1985; Newman-Tancredi et al., 1998; Millan et al., 2000).

An alternative method for selectively activating NA receptors is central administration of the neurotransmitter itself. This pharmacological approach has been previously used to examine the role of NA in behaviours such as locomotor activity and feeding. Infusion of 20-80 μ g of NA into the lateral ventricle over a 60-min period has been shown to significantly increase locomotor activity in rats (Segal and Mandell, 1970; Geyer et al., 1972). Similarly, Levine et al (1991) demonstrated that i.c.v. administration of NA (20 μ g) stimulates feeding within 5 min of its administration, and maintains this feeding behaviour for a period of time similar to that seen following food deprivation. Based on this evidence of behavioural activation, Experiment 1A was carried out to determine whether, and in what dose range, i.c.v. injections of NA would induce the reinstatement of cocaine seeking.

After establishing that central injections of NA administered in a dose range of 10-20 μ g was indeed effective in inducing reinstatement, I carried out a series of complementary molecular experiments (Experiment 1B) to determine whether NA at these doses was associated with an up-regulation in the expression of genetic markers of neuronal activation using *in situ* hybridization. This technique was selected because it provides a powerful means for quantifying and localizing changes in gene expression in individual neurons, thus providing important information about how discrete groups of neurons respond to pharmacological or physiological challenges. Specifically, I examined the expression of *c-fos* and CRF mRNA in

the CeA and BNST, brain regions known to be involved in the effects of NA on reinstatement, and regions where NA and CRF systems would be expected to interact in mediating the effects of stress on reinstatement of drug seeking (as described in the neuroanatomical model outlined in the General Introduction).

Expression of the proto-oncogene *c-fos*, which belongs to the immediate early gene family of transcription factors, is frequently used as a measure of neuronal activity because it is often expressed when neurons fire (Cullinan et al., 1995). Similarly, examination of CRF mRNA has been used effectively to detect transient changes in the activity of the system, in response to environmental or pharmacological stressors (Lightman and Young, 1989; Rivier et al., 1990). For purposes of comparison, both *c-fos* and CRF mRNA levels were also examined in the parventricular nucleus of the hypothalamus (PVN), since NA in this region is a primary regulator of CRF activity and the endocrine stress response (Swanson and Sawchenko, 1980).

The remaining experiments in this chapter were designed to more directly assess whether a functional interaction between CRF and NA systems can contribute to the reinstatement of cocaine seeking and, if so, to determine the direction of this interaction. Using the i.c.v. NA method developed in Experiment 1A, Experiment 2 explored the hypothesis that activation of postsynaptic NA receptors would induce the reinstatement of cocaine seeking by stimulating the release of CRF. This hypothesis was based on the neuroantomical evidence showing that the ventral NA projection, previously shown to be involved in stress-induced reinstatement, terminates in close proximity to CRF cell bodies in the CeA and makes direct synaptic contact with CRF cells in the ventral BNST (Hornby and Piekut, 1989; Phelix et al., 1994). In addition, it has recently been shown that β_1 -adrenoceptors are expressed on CRF-immunoreactive neurons in the CeA (Rudoy et al., 2009). Thus, I predicted that antagonism of CRF receptors would interfere with the effects of NA on

reinstatement, presumably by blocking the actions of CRF at receptors downstream from the sites of action of NA.

Although there is a sound neuroanatomical and behavioural basis to hypothesize a NA-CRF direction of interaction in mediating reinstatement of cocaine seeking, it is also possible that the opposite direction of interaction between the systems could contribute to reinstatement. That is, CRF could also potentially act to alter NA transmission either at the level of the NA cell bodies or presynaptically in NA terminal regions such as the CeA and BNST (see Figure 3). For example, it is known that the firing of NA neurons in the LC is increased by CRF (Emoto et al., 1993; Valentino et al., 1993), and it has been suggested that a similar type of interaction may occur in the lateral tegmental nuclei (Koob, 1999; Dunn and Swiergiel, 2008). In addition, it is also possible that CRF receptors located on presynaptic terminals in the CeA and BNST could conceivably alter NA transmission in these regions. To test for this alternate direction of interaction, I activated CRF receptors with central injections of the peptide while simultaneously blocking NA transmission with the α_2 -adrenoceptor agonist, clonidine (Experiment 3).

In Experiment 4, I used an alternate means of activating the NA system, namely administration of the prototypical α_2 -adrenoceptor antagonist, yohimbine, which acts at the α_2 autoreceptor to enhance NA release. The aim of this experiment was to assess whether antagonism of CRF receptors would have similar effects on yohimbine-induced reinstatement, as those seen following i.c.v. injections of NA in Experiment 1. As described previously, considerable research has been conducted in recent years, using yohimbine as a pharmacological stressor to induce the reinstatement of drug seeking to several different drugs of abuse. Given that yohimbine acts as a potent activator of NA transmission, and given the important role NA is known to play in stress-induced reinstatement of drug seeking, there is

good reason to hypothesize that yohimbine induces reinstatement of drug seeking via its effects on NA transmission. Moreover, it is plausible that this effect of yohimbine on NA transmission might ultimately lead to reinstatement of drug seeking via a subsequent interaction with CRF systems. Consistent with these hypotheses, it has previously been shown that pretreatment with the selective CRF₁ receptor antagonist, antalarmin, significantly attenuates the reinstatement of yohimbine-induced alcohol (Marinelli et al., 2007) and food (Ghitza et al., 2006) seeking in rats. These effects, however, have not previously been extended to animals with a history of cocaine self-administration.

Experiment 1A: Effects of i.c.v. NA on the reinstatement of cocaine seeking Materials and Methods

Subjects

Nine male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and NA bitartrate (Sigma-Aldrich) were both dissolved in sterile, physiological saline. The vehicle used in these experiments was physiological saline. As previously described in the introduction of this chapter, central injections of NA at doses of 10 and 20 µg was selected based on previous reports showing increased locomotor activity and feeding behaviour within this dose range (Segal and Mandell, 1970; Geyer et al., 1972; Levine et al., 1991). Central injections of NA were conducted according to the microinjection procedures described in the Microinjection Procedures section of the General Methods (Chapter 2).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in the Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in Chapter 2 (also see Figure 4), with the exception that after the first extinction session on Day 3, animals were given a mock i.c.v. injection, and the second extinction session began 5 min after this injection.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for NA-induced reinstatement over 3 consecutive days as described in the General Methods (see Figure 4). Briefly, at the time of testing for reinstatement, animals were administered an i.c.v. injection of NA (0, 10, 20 μ g). Five minutes following the i.c.v. injection, tests for reinstatement began, whereby the previously drug-reinforced lever was extended into the chamber and responding was recorded over a 180-min period. Each animal was tested with all doses of NA (0, 10, 20 μ g) on alternate days, in a counterbalanced order.

Results

Self-administration Training

Once animals acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 27.7 \pm 4.33 and 27.75 \pm 4.63 infusions in each 180-min session, corresponding to a total intake of 6.37 \pm 1.06 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever (compared to the last 2 days of cocaine self-administration), averaging 58.2 ± 16.77 responses in the first 60-min extinction session. This rate of responding gradually declined to a low 8.9 ± 2.4 active lever responses in the final 60-min session on Day 3 of extinction training (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).





Representative acquisition of cocaine self-administration over the training days.





Representative responding during hourly extinction sessions over the 3-day extinction training phase.

Testing for Reinstatement

Figure 8 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers in each hour of the 180-min tests for NA-induced reinstatement. It can be seen that most responses on the active lever (Fig 8A) occurred in the first hour of testing. Moreover, animals responded more after administration of 10µg or 20µg of NA than after vehicle during this first hour. These observations were confirmed by a repeated measures ANOVA which revealed significant main effects of Test Condition (*F*[2,14] = 6.9, *p*<0.01) and Hour of Testing (*F*[2,14]=25.6, *p*<0.001), and a significant Test Condition by Hour interaction (*F*[4,28]=8.5, *p*<0.001). Subsequent repeated measures ANOVAs for each hour revealed that the two-way interaction was attributable to a significant effect of Test Condition only in the first hour of testing (*F*[1, 6] = 10.9, *p*<0.02), and that in this first hour, both doses of NA (10 and 20 µg) relative to vehicle were associated with a significantly greater number of responses on the previously active lever.

Figure 8B shows that responding on the inactive lever was very low during all test sessions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Hour (F[1,7]=14.98, p<0.01), reflecting slightly higher responding in the first than second or third hours of testing, there was no effect of Test Condition, and no interaction between the factors (p's>0.1).

Figure 8



Mean (±SEM) number of responses on the active (A) and inactive (B) levers in the 180-min tests for NA-induced reinstatement after injection of VEH (n=9) or NA (10 [n=9] or 20 µg [n=9] µg, i.c.v.).

* NA (10 and 20 µg) different from VEH, *p*<0.02

Experiment 1B: Effects of i.c.v. NA on *c-fos* and CRF mRNA expression in the CeA, BNST and PVN

Materials and Methods

Subjects

Thirty-six male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with an i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Procedures

After a 7-10 day recovery period following surgery, drug naïve rats were handled for 5 days. On days 4 and 5 of handling, animals received sham i.c.v. injections in their home cage to habituate animals to subsequent test procedures. Twenty-four hours after the last sham i.c.v. injection, animals were challenged with an i.c.v. injection of either dose of NA (10 or 20 µg) or its vehicle (saline). Forty-five or 90 min following the i.c.v. injection, animals were briefly (30-45 s) exposed to isoflurane vapors before being killed by decapitation. The brain was rapidly removed, flash-frozen in isopentane (-35 to -40°C) and stored at -86°C until processing for *in situ* hybridization. Tissue sectioning, *in situ* hybridization and autoradiography procedures were carried out as described in the General Methods (Chapter 2).

A 45- or 90-min delay following central injection of the test substance was employed because of the known kinetics of *c-fos* and CRF transcription, where transient increases in *c-fos* mRNA expression peaks approximately 30 min following exposure to acute stimuli and CRF mRNA subsequently peaks approximately 30-45 min later (Imaki et al., 1991; Imaki et al., 1993).

Results

Effects of i.c.v. NA on c-fos mRNA in CeA, BNST and PVN

Figure 9 shows mean (\pm SEM) *c-fos* mRNA (µCi/g) levels in dBNST (A), vBNST (B), CeA (C), and PVN (D) of tissue collected 45 or 90 min following i.e.v. injection of NA or its vehicle. It is readily apparent that any effects of NA on *c-fos* mRNA expression were restricted to tissues collected 45 min after injection; i.e., there were no detectable changes in *cfos* expression induced by NA in any brain region after 90 min (all *p*'s >0.1). In animals killed 45 min after injection, separate univariate ANOVAs for each brain area revealed significant effects of Test Condition in dBNST (*F*[2,15] = 13.53, *p*<0.001), CeA (*F*[2, 14] = 3.8, *p*<0.05), and PVN (*F*[2,15] = 8.418, *p*<0.001). In all cases, subsequent post-hoc comparisons revealed that both doses of NA were associated with a higher level of *c-fos* mRNA expression relative to vehicle and, in the dBNST, there was an additional significant difference in *c-fos* expression between the 10 and 20 µg conditions (*p*'s <0.05). In the vBNST, the effect of Test Condition was non-significant. Representative photomicrographs for each region of interest are shown in Figure 10.

Effects of i.c.v. NA on CRF mRNA in CeA, BNST and PVN

Figure 11 shows mean (\pm SEM) CRF mRNA (μ Ci/g) levels in dBNST (A), vBNST (B), CeA (C), and PVN (D) of tissue collected 45 or 90 min following i.c.v. injection of NA or its vehicle. As can be seen in the figure, there were no detectable changes in CRF mRNA expression induced by NA in any brain region after a 45 min (all *p*'s >0.4) or 90 min (all *p*'s





Mean (\pm SEM) *c-fos* mRNA (μ Ci/g) levels in dBNST (A), vBNST (B), CeA (C), and PVN (D) of tissues collected 45 (n = 18) or 90 min (n = 18) following injection of VEH or NA (10 or 20 μ g, i.c.v.).

* NA (10 and 20 μ g) different from VEH, *p*'s <0.05; ** NA (20 μ g) different from NA (10 μ g) and VEH, *p*<0.05

Figure 10



Left panels (A-C): plates modified from a brain atlas (Paxinos and Watson, 1998) illustrating the sampling area where measurements of *c-fos* and CRF mRNA were made in the (A) dBNST (light grey) and vBNST (dark grey), (B) CeA and (C) PVN. Right panels (D-F): representative autoradiograms demonstrating localization of *c-fos* mRNA in the dBNST, vBNST, CeA and PVN.





Mean (\pm SEM) CRF mRNA (μ Ci/g) levels in dBNST (A), vBNST (B), CeA (C), and PVN (D) of tissues collected 45 (*n*=18) or 90 min (*n*=18) following VEH or NA (10 or 20 μ g, i.c.v.).
>0.06) delay. Although the univariate ANOVA analyses conducted on the 90 min data did not reveal any significant increases in CRF mRNA expression following administration of NA, it merits mention that the 10 µg dose of NA was associated with a modest, albeit non-significant, increase in CRF mRNA expression in the CeA (F[2,15] = 34.86, p=0.06). Indeed, post-hoc comparison reveals that the 10 µg dose of NA was associated with a higher level of CRF mRNA expression relative to vehicle (t[10]=2.22, p<0.03).

Experiment 2: Effects of CRF antagonist D-Phe CRF₁₂₋₄₁ on NA-induced reinstatement of cocaine seeking

Materials and Methods

Subjects

Eighteen male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals), NA bitartrate (Sigma-Aldrich), and the CRF receptor antagonist, CRF D-Phe₁₂₋₄₁ (Bachem), were all dissolved in sterile, physiological saline. Both NA and D-Phe were centrally administered according to the microinjection procedures described in the General Methods (Chapter 2). The vehicle used in all pretreatment and test challenge conditions in this experiment was physiological saline.

The dose of NA (10 μ g) was selected based on the experimental outcome of Experiment 1A in this chapter. Similarly, the dose of D-Phe (1 μ g) was justified based on the results of previous reinstatement studies conducted in our lab, demonstrating that at this dose, administration of the antagonist blocks footshock-induced reinstatement of extinguished cocaine seeking (Erb et al., 1998; see General Introduction for a complete discussion of these results).

Reinstatement Procedures

Phase 1: Cocaine Self-administration

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also see Figure 4), with the exception of the timing of sham i.c.v. injections. After the first extinction session on Day 3, animals were given 2 sham i.c.v. injections separated by 30-min. The second extinction session began 5 min after the sham injection.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase. Subsequently, animals were given central injections of the non-selective CRF receptor antagonist, D-Phe (0, 1 μ g; i.c.v), 30-min prior to central injections of NA (0, 10 μ g; i.c.v). Five minutes following the NA i.c.v. injection, tests for reinstatement began, whereby the previously drug-reinforced lever was extended into the chamber and responding was recorded over a 60-min period (test sessions were reduced to 60-min in length based on the results of Experiment 1A which showed that most of the responding occurred in the first hour of test). Different groups of animals were pretreated with the 0 and 1 μ g dose of D-Phe, but each animal was tested after NA (10 μ g) and its vehicle on alternate days, in a counterbalanced order.

Results

Self-administration Training

Once animals acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was 32.97 ± 2.07 and 31.12 ± 2.05

infusions in each 180-min session, corresponding to a total intake of 7.16 ± 0.47 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever (compared to the last 2 days of cocaine self-administration), with the D-Phe pretreatment group averaging 52.07 ± 9.13 responses, and vehicle pretreatment group averaging 45.11 ± 14.34 responses in the first 60-min extinction session (i.e., Extinction Day 1, session 1). Responding rates for animals assigned to the D-Phe pretreatment group, gradually declined to a low 9.71 ± 5.58 and 3.14 ± 0.74 responses in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Similarly, animals assigned to the vehicle pretreatment group declined to a low 7.11 ± 1.81 and 4.44 ± 1.31 responses in the extinction session immediately prior to reinstatement test 1 and 2, respectively (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 12 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals were pretreated with D-Phe (0, 1 µg) and challenged with i.c.v. injections of VEH or NA (10 µg) prior to testing. It can be seen in Figure 12A that NA induced a higher level of responding on the active lever than did its VEH, in animals pretreated with 0 µg dose D-Phe, but not in animals pretreated with D-Phe (1 µg). Thus, pretreatment with D-Phe blocked the reinstatement of responding induced by NA. A repeated measures ANOVA confirmed these observations by revealing a significant

Figure 12



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with D-Phe (0 [*n*=8], 1 µg [*n*=10]; i.c.v.) and challenged with VEH or NA (10 µg; i.c.v.) prior to testing.

* Different from all other conditions, p < 0.05.

main effect of Test Challenge (F[1, 16]=7.88, p<0.03), and interaction of Pretreatment by Test Challenge (F[1,16]=6.29, p<0.03). Separate paired samples *t*-tests for each pretreatment revealed a higher level of responding in the NA relative to VEH condition, in animals pretreated with 0 µg dose D-Phe but not D-Phe (1 µg; p<0.05). Likewise, separate independent samples *t*-tests for each Test Challenge revealed a higher level of responding following NA injection in 0 µg dose D-Phe relative to D-Phe (1 µg) pretreated animals (p<0.05).

As can be seen in Figure 12B, responding on the inactive lever was very low (less than 5 responses) across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,16]=5.92, p<0.03), reflecting slightly higher responding following i.c.v. NA than its vehicle, there was no interaction of Pretreatment by Test Condition (p>0.1).

Experiment 3: Effects of clonidine on CRF-induced reinstatement of

cocaine seeking

Materials and Methods

Subjects

Nineteen male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals), CRF (Sigma-Aldrich) and clonidine (Sigma-Aldrich) were all dissolved in sterile, physiological saline. The vehicle used in all pretreatment and test challenge conditions was physiological saline. CRF was centrally administered according to the microinjection procedures described in the General Methods (Chapter 2).

The dose of CRF (0.5 μ g, i.c.v.) was justified on the basis of previous reinstatement studies, showing that administration of the neuropeptide at this dose is effective in inducing robust and reliable reinstatement (Erb et al., 1998; Erb et al., 2006; Mantsch et al., 2010). Similarly, the dose of clonidine (40 μ g/kg, i.p.) was selected based on previous reports showing that pretreatment of the drug in the dose range of 20-40 μ g/kg, i.p., blocks footshockinduced reinstatement of cocaine and heroin seeking in rats (Erb et al., 2000; Shaham et al., 2000b).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in Chapter 2 (also see Figure 4), with the exception of the timing and route of sham/saline injections. After the first extinction session on Day 3, animals were given an i.p. injection of saline, followed 30-min later by a sham i.c.v. injection. The second extinction session began 15 min after the i.c.v. injection.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase. Subsequently, animals were given a systemic injection of the α_2 adrenoceptor agonist clonidine (0, 40 µg/kg; i.p) 30-min prior to an i.c.v injection of CRF (0, 0.5 µg). Fifteen minutes following the administration of CRF, tests for reinstatement began whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with the 0 or 40 µg/kg doses of clonidine, but each animal was tested with CRF (0.5 µg) and its vehicle on alternate days, in a counterbalanced order.

Results

Self-administration Training

Once animals acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period (see Figure 6). The mean (\pm SEM) number of infusions made on the last 2 days of training was 37.56 \pm 2.12 and 38.37 \pm 2.30 infusions in each 180-min session, corresponding to an intake of 8.83 \pm 0.53 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever (compared to the last 2 days of cocaine self-administration), with the clonidine pretreatment group averaging 83.80 ± 16.37 and the vehicle pretreatment group averaging 91.12 ± 15.43 responses in the first 60-min extinction session (i.e., Extinction Day 1, session 1). Responding rates for animals assigned to the clonidine pretreatment group, gradually declined to a low 9.7 ± 9.39 and 4.40 ± 3.0 in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Similarly, animals assigned to the vehicle pretreatment group declined to a low 12.57 ± 4.44 and 4.86 ± 1.94 in the extinction session immediately prior to reinstatement test 1 and 2, respectively (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 13 shows the mean (<u>+</u>SEM) number of responses on the active (A) and inactive (B) lever during the 60-min tests for reinstatement in which animals were pretreated with

clonidine (0, 40 µg/kg) and challenged with i.c.v. injections of VEH or CRF (0.5 µg) prior to testing. Central injections of CRF (0.5 µg) reinstated responding on the active lever, and pretreatment with clonidine (40 µg/kg) did not attenuate this effect (Figure 13A). The repeated measures ANOVA revealed only a significant main effect of Test Challenge (*F*[1,17]=43.46, p<0.001). The Pretreatment by Test Challenge interaction was not significant (*F*[1, 17]=12.84, p>0.5).

As can be seen in Figure 13B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,18]=12.86, p<0.002), reflecting higher responding following i.c.v. CRF than its vehicle, there was no interaction of Pretreatment by Test Condition (p>0.6).

Experiment 4: Effects of CRF antagonist D-Phe CRF₁₂₋₄₁ on yohimbine-induced reinstatement of cocaine seeking

Materials and Methods

Subjects

Thirty four male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Figure 13



Mean (\pm SEM) number of responses on the active (A) and inactive (B) lever during the 60-min tests for reinstatement in which animals were pretreated with clonidine (0 [*n*=10], 40 µg/kg [*n*=9]; i.p.) and challenged with VEH or CRF (0.5 µg; i.c.v.) prior to testing. * CRF different from VEH test challenge, *p*<0.05.

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and the CRF receptor antagonist, CRF D-Phe₁₂₋₄₁ (Bachem), were both dissolved in sterile, physiological saline. The α_2 -adrenoceptor antagonist, yohimbine (Sigma-Aldrich), was dissolved in distilled water. D-Phe was centrally administered according to the microinjection procedures described in the General Methods (Chapter 2). The vehicle administered in the pretreatment condition was saline, whereas the vehicle administered in the test challenge condition was distilled water.

The dose of yohimbine (1.25 mg/kg, i.p.) used in this study was selected on the basis of dose-response studies carried out in my laboratory and the work of others (Shepard et al., 2004; Le et al., 2005; Feltenstein and See, 2006; Ghitza et al., 2006; Fletcher et al., 2008), indicating comparable levels of reinstatement by yohimbine in the 1.25-2.5 mg/kg, i.p. dose range.

Reinstatement Procedures

Phase 1: Cocaine Self-administration

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also see Figure 4), with the exception of the timing and route of sham/saline injections. In this experiment, acclimatization procedures involved a sham i.c.v. injection, followed 30-min later by an i.p. injection of distilled water. The second extinction session began 30 min after the i.p. injection.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase. Subsequently, animals were given central injections of D-Phe (0, 1, 5 μ g; i.c.v) 30-min prior to systemic injections of yohimbine (0, 1.25 mg/kg; i.p.). Thirty minutes following the yohimbine injection, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with the 0, 1, or 5 μ g dose of D-Phe, but each animal was tested with yohimbine (1.25 mg/kg) and its vehicle on alternate days, in a counterbalanced order.

Results

Self-administration Training

Once animals acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 33.03 ± 2.07 and 31.12 ± 2.05 infusions in each 180-min session, corresponding to an intake of 7.18 ± 0.47 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever (compared to the last 2 days of cocaine self-administration), with the D-Phe (1 μ g) pretreatment group averaging 88.81±12.34, D-Phe (5 μ g) pretreatment group averaging 69.11±13.21 and the vehicle pretreatment group averaging 84.67±21.79 responses in the first 60-min extinction session (i.e., Extinction Day 1, session 1).

Responding rates for animals assigned to the D-Phe (1 μ g) pretreatment group, gradually declined to a low 9.56±1.89 and 4.25±1.30 in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Similarly, animals assigned to the D-Phe (5 μ g) pretreatment group declined to a low 10.67±2.80 and 5.89±3.45 in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Finally, animals assigned to the vehicle pretreatment group declined to a low 5.11±1.31 and 4.66±1.67 in the extinction session immediately prior to reinstatement test 1 and 2, respectively (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 14 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) lever during the 60-min tests for reinstatement in which animals were pretreated with D-Phe (0, 1, 5 µg; i.c.v.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing. Examination of Figure 14A shows that pretreatment with the different doses of D-Phe (0, 1 or 5 µg) was without effect on reinstatement of cocaine seeking induced by yohimbine. The repeated measures ANOVA revealed only a main effect of Test Challenge (*F*[1,31]=13.51, *p*<0.001).

As can be seen in Figure 14B, responding on the inactive lever was very low (less than 10 responses) across all pretreatment and test challenge conditions. A repeated measures ANOVA for responses on this lever revealed no effect of Test Challenge on the number of responses made on the inactive lever during tests for reinstatement (p>0.1) as well as no interaction of Pretreatment by Test Condition (p>0.8).

Experiment 5: Effects of CRF antagonist D-Phe CRF₁₂₋₄₁ on CRF-induced reinstatement (Validation Study)

The results of Experiment 4 unexpectedly showed that the CRF antagonist D-Phe CRF₁₂₋₄₁ did not block the effects of yohimbine on the reinstatement of cocaine seeking. One possible explanation for these negative findings was that the preparation of the antagonist that I was working with was not biologically active. Thus, Experiment 5 was conducted to rule out this possibility by re-testing animals from Experiment 4 to determine whether the same stock of D-Phe would block CRF-induced reinstatement.

Figure 14



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with D-Phe (0 [*n*=9], 1 [*n*=16], 5 µg [*n*=9]; i.c.v.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing. * Yohimbine different from VEH test challenge, *p*<0.01.

Materials and Methods

Subjects

Eleven animals from Experiment 4 in this chapter were used for this validation study.

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

CRF (Sigma-Aldrich) and CRF D-Phe₁₂₋₄₁ (Bachem) were dissolved in sterile, physiological saline, and administered according to the microinjection procedures described in the General Methods (Chapter 2). The selected doses of both drugs were the same as those described in Experiments 2-4. The vehicle used in all pretreatment and test challenge conditions in this experiment was physiological saline.

Procedures For Re-Testing for Reinstatement

Phase 1: Extinction Training

Approximately 7 days following the final test for reinstatement conducted in Experiment 4, animals were re-extinguished and re-acclimatized to test procedures. Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4), with the exception of the timing of sham i.c.v. injections. After the first extinction session on Day 3, animals were given 2 sham i.c.v. injections separated by 30-min. The second extinction session began 30-min after the second sham injection.

Phase 2: Re-testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase, until the number of responses on the active lever was 15 or fewer. Once this criterion was reached, animals were given central injections of D-Phe (0, 1 μ g; i.c.v) 30 min prior to an i.c.v injection of CRF (0, 0.5 μ g). Thirty minutes following the CRF injection, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with either 0 or 1 μ g D-Phe, but each animal was tested with both CRF (0.5 μ g) and its vehicle on alternate days, in a counterbalanced order.

Results

Testing for Reinstatement

Figure 15 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) lever during 60-min tests for reinstatement in which animals had been pretreated with D-Phe (0, 1 µg) and challenged with VEH or CRF (0.5 µg) prior to testing. It can be seen in Figure 15A that CRF (0.5 µg) induced a higher level of responding than did its vehicle in animals pretreated with the 0 µg dose of D-Phe, but not in animals pretreated with the 1 µg dose of D-Phe. A repeated measures ANOVA revealed a Pretreatment by Test Challenge interaction that reached significance (*F*[1,9]=4.96, *p*=0.05), and post hoc comparisons confirmed an attenuating effect of D-Phe on CRF-induced reinstatement.

Figure 15



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with D-Phe (0 [n=5], 1 µg [n=6]; i.c.v.) and challenged with VEH or CRF (0.5 µg; i.c.v.) prior to testing.

As can be seen in Figure 15B, responding on the inactive lever was low during all test sessions. A repeated measures ANOVA for responses on this lever revealed no main effect of Test Challenge (p>0.1) and no interaction of Pretreatment by Test Condition (p>0.6).

Discussion

Four major findings emerge from the series of experiments presented in this chapter. First, direct activation of NA receptors, by i.c.v. NA, induced the reinstatement of cocaine seeking. Second, doses of NA associated with the induction of reinstatement also induced reliable neuronal activation (as reflected in increases in *c-fos* mRNA expression) within brain regions previously implicated in the effects of NA on reinstatement of cocaine seeking. Unexpectedly, however, these effects of NA on neuronal activation did not extend to increases in the expression of CRF mRNA in these regions. Third, it was shown that NA and CRF interact to induce reinstatement of cocaine seeking, in a manner suggesting that the effects of CRF occur subsequent to and downstream of the effects of NA. Finally, it was unexpectedly found that antagonism of CRF receptors did not interfere in yohimbine-induced reinstatement of cocaine seeking.

Central injections of NA induce the reinstatement of cocaine seeking

The first novel finding reported here, that central injections of NA (10, 20 μ g) reliably reinstate extinguished cocaine seeking in rats after extended drug-free periods (see Figure 8), is consistent with previous reports concerning a role for NA in stress-induced reinstatement. For example, pretreatment with the α_2 -adrenoceptor agonists clonidine, lofexidine and guanabenz, prior to tests for reinstatement, attenuates footshock-induced reinstatement of extinguished heroin (Shaham et al., 2000b) and cocaine (Erb et al., 2000) seeking in rats, and does so by decreasing NA transmission in the ventral NA pathway (Shaham et al., 2000b).

Clearly, the effect of NA on reinstatement of cocaine seeking is modest when compared to the reinstatement effects associated with other forms of stress, such as footshock stress, i.c.v. injections of CRF, or systemic injections of vohimbine (e.g., Erb et al., 1996; Shepard et al., 2004; Mantsch et al., 2008). Indeed, these other stressors, though often variable with respect to the magnitude of their effects across studies, are quite commonly associated with reinstatement response rates upwards of 30-40 responses in a 60-min test session. By comparison, i.c.v. NA resulted in 12-15 responses in a 60-min period. Although it is not clear why NA induces a comparatively weaker effect on reinstatement responding compared with other forms of stress, a reasonable explanation most likely involves a short duration of action of the transmitter in the central nervous system. Indeed, NA is rapidly removed from the synapse via reuptake by the NA transporter (NET) and metabolically degraded by the two major catecholamine enzymes monamine oxidase (MAO) and catechol-O-methyl-transferase (COMT) (Glowinski and Axelrod, 1966; Axelrod, 1971). Whatever the reasons for the modesty of the effect, however, it warrants mention that it is an effect that is highly statistically reliable. Moreover, based on my success in obtaining the effect in more than one experiment, it is an effect that is reproducible and reliable.

Central injections of NA induce increases in c-fos, but not CRF, mRNA expression within brain regions known to mediate the effects of stress on reinstatement of cocaine seeking

A second important finding of this series of experiments is that doses of NA associated with the reliable induction of reinstatement of cocaine seeking also induce neuronal activation within brain regions previously implicated in the effects of NA on reinstatement of cocaine seeking. These include CeA and BNST. More specifically, in Experiment 1B, I studied the effects of i.c.v. NA on the expression of *c-fos* mRNA, an immediate early gene, whose up-regulation is associated with recent neuronal activation (Cullinan et al., 1995). The results of this experiment revealed a significant increase in *c-fos* mRNA levels in the CeA and dorsal subregion of the BNST 45 min, though not 90 min, following central injections of NA (10, 20 µg; see Figure 9). This time dependent change in *c-fos* expression is consistent with the known kinetics of *c-fos* transcription, where transient increases in *c-fos* mRNA expression peaks approximately 30 min following exposure to acute stimuli (Imaki et al., 1993; Chaudhuri et al., 2000). Thus, these findings corroborate previous findings concerning a role for NA in reinstatement of cocaine seeking by demonstrating that direct activation of NA receptors subsequently activates a population or populations of neurons in CeA and BNST, regions implicated in the effects of NA on reinstatement of cocaine seeking (Leri et al., 2002).

One somewhat perplexing aspect of the findings in Experiment 1B is the selective *c-fos* mRNA response to NA in the dorsal, but not ventral, region of the BNST. The BNST is an anatomically and functionally heterogeneous brain region, with respect to the distributions of its afferent and efferent projections (Alheid and Heimer, 1988). As mentioned, the BNST is a major target of NA transmission in the brain, with NA innervation particularly prominent in its ventral and medial subdivisions (Brownstein et al., 1974; Versteeg et al., 1976). Thus, given the neuroanatomical organization of NA inputs to the BNST, and the comparatively less abundant input to the dorsal than ventral subregions, it may seem surprising that it was the dorsal region that selectively expressed elevated levels of *c-fos* mRNA. On the other hand, the lack of effect of NA on *c-fos* mRNA expression in the ventral BNST may reflect an inhibitory effect of the transmitter in this region. For example, NA (10 or 100 μM) in the ventral BNST has been found to trigger inhibitory postsynaptic potentials in this region by GABA_A receptors

(Dumont and Williams, 2004). Thus, it is conceivable that the effects of NA in this region, in contrast to the dorsal BNST and CeA, are primarily inhibitory. Consistent with these findings, (Casada and Dafny, 1991) reported that in 70% of neurons from which they recorded in the BNST, iontophoretic application of NA resulted in a decrease in firing rate, and this effect was particularly evident in the ventral BNST. Thus, the lack of *c-fos* response in the ventral BNST that was observed in Experiment 1B may reflect a lack of excitatory activity of neurons in this region rather than an absence of neuronal activity. Indeed, the accuracy of *c-fos* mRNA as a measure of neuronal activity of neurons under net synaptic inhibition is unclear and the subject of some debate (Kovacs, 2008).

Finally, in Experiment 1B, the NA manipulation was also associated with a robust induction of *c-fos* mRNA in the PVN. This result was not at all surprising, given that NA in the PVN is known to initiate the cascade of events involving activation of the HPA axis in response to stress (Swanson and Sawchenko, 1980; Pacak et al., 1995). It is important to note, however, that while providing an important positive control for the present study, it was not expected that this increase in *c-fos* mRNA expression would correlate with the behavioral effects of NA on reinstatement of cocaine seeking. In fact, as mentioned previously, there is considerable evidence showing that stress-induced reinstatement of drug seeking is not dependent on an intact HPA response. For example, andrenalectomy does not attenuate stress-induced reinstatement in heroin-trained rats (Shaham et al., 1997), and footshock is equally effective in inducing the reinstatement of cocaine seeking in adrenalectomized rats given corticosterone replacement and sham adrenalectomized animals (Erb et al., 1998).

As mentioned in the introduction of this Chapter, it was expected that i.c.v. NA would not only induce neuronal activation in CeA and BNST, but that it would also result in an decrease in CRF mRNA expression in these regions. This prediction was based on

neuroanatomical data demonstrating that DA β -hydroxylase (DBH)-immunoreactive fibers synapse directly onto CRF-containing perikarya in the ventromedial BNST (Hornby and Piekut, 1989; Phelix et al., 1994) and that, in the CeA, CRF- immunoreactive neurons occur in close proximity to NA terminals (Hornby and Piekut, 1989) and express β_1 -adrenoceptors (Rudoy et al., 2009). In addition, the prediction was based on findings that CRF mRNA is upregulated in BNST and CeA in response to footshock stress (Funk et al., 2006), and that the effect of this stressor on reinstatement is mediated, at least in part, by the actions of NA and CRF in these regions (Erb and Stewart, 1999; Leri et al., 2002). Finally, pretreatment with the β_1 -adrenoceptor antagonist, betaxolol, during early cocaine withdrawal has been found to decrease CRF mRNA expression in the amygdala (Rudoy et al., 2009).

Despite these substantial corroborative findings, I failed to show a NA-induced change in CRF mRNA expression in the CeA, BNST or PVN in Experiment 1B. One possible explanation for this null finding is that NA, at the doses administered, was sufficient to induce neuronal activation, but insufficient to produce an increase in CRF synthesis. Once injected, NA is rapidly metabolized and removed from the extracellular fluid by NA transporters (Iversen et al., 1966; Axelrod, 1971), and this homeostatic process may limit the amount of time NA has to stimulate the extrahypothalamic stress response. Consequently, while NA may have activated CRF-containing cells to induce the release of CRF (as suggested by increases in *c-fos* mRNA expression), it may have failed to activate those neurons to such a level that CRF stores were depleted and increased transmitter synthesis was induced. Similar dissociations between *c-fos* and CRF mRNA expression following exposure to stress have been previously reported. For example, significant increases in *c-fos* mRNA in the PVN following exposure to somatovisceral pain induced by 2% acetic acid is not accompanied by increases in CRF mRNA expression in the PVN or CeA (Hwang et al., 2007). It is worth noting that an increase in CRF mRNA expression by NA approached significance in the CeA, a region in which Leri and colleagues (2002) reported a blockade of the effects of footshock on reinstatement by a $\beta_{1/2}$ - adrenoceptor antagonist cocktail. In contrast, the attenuating effect of the $\beta_{1/2}$ - adrenoceptor manipulation in the BNST was less robust. Taken together, these findings suggest that NAergic signaling via adrenoceptors located on CRF containing neurons (Rudoy et al., 2009) may be especially important in the CeA. Of course this hypothesis is highly speculative, and would need to be substantiated with evidence from, for example, double labeling studies showing the pattern of connectivity between NA and CRF systems in the CeA versus the BNST.

It is important to note that all *in situ* hybridization studies were carried out in drug naïve rats. Although these systems are likely to interact with each other regardless of drug history, there is evidence to suggest that the strength of the *c-fos* and CRF mRNA expression may have been stronger in animals that had self-administered cocaine and undergone extinction. For example, it has been shown that footshock enhances CRF mRNA expression in the amygdala and dorsal BNST of animals with a history of heroin, but not sucrose, self-administration (Shalev et al., 2001). From this perspective, it may have been expected that a change in these parameters may have resulted in a stronger effect of i.c.v. NA on CRF mRNA.

NA and CRF interact to mediate reinstatement of cocaine seeking, and the effects of CRF occur subsequent and downstream of the effects of NA

Although i.c.v. NA was ineffective in increasing CRF mRNA expression in Experiment 1B, the critical roles that both NA and CRF systems play in the effects of stress on reinstatement of cocaine seeking, along with compelling anatomical evidence showing an interaction between the systems in brain regions where they are known to mediate stressinduced reinstatement, justified a rationale for directly exploring the idea that an interaction between NA and CRF systems contributes to the reinstatement of cocaine seeking. To this end, in Experiment 2, I used the parameters established in Experiment 1A to activate NA receptors while, simultaneously, antagonizing CRF receptors. The results of this experiment confirmed my hypotheses and the neuroanatomical model of stress-induced reinstatement of drug seeking detailed in Chapter 1. Specifically, the results were consistent with the idea that CRF acts subsequent to and downstream of the effects of NA to induce reinstatement; indeed, pretreatment with the CRF antagonist, D-Phe CRF₁₂₋₄₁, completely blocked NA-induced reinstatement.

In order to clarify the specificity of the direction of NA-CRF interactions in mediating reinstatement, Experiment 3 was carried out to explore the possibility that NA may also act subsequent to the actions of CRF in mediating reinstatement. To test for this possibility, CRF receptors, at the time of testing for reinstatement, were activated with central injections of the peptide while, simultaneously, blocking NA transmission with the α_2 -adrenoceptor agonist, clonidine. In this case, pretreatment with clonidine failed to block CRF-induced reinstatement of cocaine seeking, arguing against the idea that that a CRF-NA direction of interaction contributes to reinstatement of cocaine seeking.

Although the combined outcomes of Experiment 2 and 3 *favor* a NA-CRF direction of interaction in the reinstatement of cocaine seeking, they do not rule out the possibility that the alternate direction of interaction might also be involved. Indeed, although the effects of clonidine in Experiment 3 were non-significant, visual inspection of Figure 13 reveals what would seem an arguably modest attenuation in CRF-induced responding in the clonidine relative to vehicle pretreatment condition. Although there are in fact several examples of NA acting downstream of CRF to mediate behavioral measures of anxiety (Yang and Dunn, 1990;

Gorman and Dunn, 1993), it is unlikely that this direction of interaction contributes in a functionally significant way to the reinstatement of drug seeking. In addition to the complete lack of statistical significance for the effects of clonidine on CRF-induced reinstatement of cocaine seeking in Experiment 3 (owing in part to the fact that clonidine produced a similar attenuating effect in the vehicle and CRF test condition), i.c.v. injections of D-Phe, administered at a dose known to block footshock-induced reinstatement of cocaine seeking (1 µg, i.c.v.), have been shown to be without effect on footshock-induced NA release in the BNST, a region in which NA is known to mediate the effects of footshock on reinstatement (Erb, Rajabi & Stewart, unpublished data).

CRF does not mediate the reinstatement of cocaine seeking by yohimbine

Based on the combined outcomes of Experiments 2 and 3, Experiment 4 was carried out to determine whether manipulations of CRF receptors would also interfere in the reinstatement of cocaine seeking induced by yohimbine, a prototypical α_2 adrenoceptor antagonist that activates NA systems. Specifically, I studied the effects of central injections of D-Phe on the reinstatement of cocaine seeking induced by systemic injections of yohimbine. In addition to the findings in Experiments 2 and 3, a prediction that antagonism of CRF receptors would interfere in yohimbine-induced reinstatement was based on two other findings: (1) recent reports that pretreatment with the CRF₁ antagonist, antalarmin, attenuates yohimbineinduced reinstatement of both alcohol (Marinelli et al., 2007) and food (Ghitza et al., 2006) seeking, and (2) findings that the reinstatement of cocaine seeking is induced in squirrel monkeys by yohimbine, and that this effect of yohimbine is blocked by pretreatment with clonidine (Lee et al., 2004). Contrary to my predictions, pretreatment with the CRF receptor antagonist failed to interfere in the reinstatement of cocaine seeking. One possible explanation for this is that I did not administer sufficiently high doses of D-Phe or, alternatively, the dose of yohimbine I tested was too high to be affected by manipulations of CRF receptors. It is unlikely, however, that either of these explanations is plausible. First, the lower dose of D-Phe (1.0 μ g) that I tested is sufficient to completely block the effects of footshock (Erb and Stewart, 1999) and NA (Experiment 2) on the reinstatement of cocaine seeking, and the 5-fold higher dose of D-Phe that I administered also failed to interfere in the effects of yohimbine on reinstatement. Moreover, in order to rule out the possibility that the D-Phe I was administering was for some reason not biologically active, I re-tested animals from Experiment 4 with the same stock of antagonist to verify, at a dose of 1 μ g, its effectiveness in interfering in the effects of CRF (0.5 μ g, i.c.v.) on reinstatement. In this test, D-Phe clearly blocked the effect of CRF (Figure 15).

It is also unlikely that the dose of yohimbine that I tested would have been insensitive to manipulations of CRF receptors, should those manipulations have been of functional significance in mediating its effects on reinstatement. In dose response studies carried out by myself (unpublished findings) and others (Shepard et al., 2004; Feltenstein and See, 2006), doses of yohimbine ranging between 0.625 and 2.5 mg/kg are associated with comparable levels of responding during tests for reinstatement, and 1.25 mg/kg is a standard challenge dose used in reinstatement testing. In fact, 1.25 mg/kg was the dose administered in the study cited previously in which pretreatment with antalarmin attenuated yohimbine-induced reinstatement of alcohol seeking (Marinelli et al., 2007).

A more likely explanation for the lack of effect of D-Phe in Experiment 4 is that yohimbine-induced reinstatement is mediated by some system other than NA. This is a possibility that formed the rationale for experiments comprising Chapter 5 of this dissertation.

CHAPTER 4

Interactions Between DA and CRF in the Reinstatement of

Cocaine Seeking

Chapter 4: Interactions Between DA and CRF in the Reinstatement of

Cocaine Seeking

It has recently been established that DA, in addition to CRF and NA, plays an important role in stress-induced reinstatement of drug seeking. As described in Chapter 1, there is evidence that activation of D₁-like receptors in PFC, most likely facilitated by enhanced DA transmission from neurons originating in VTA, mediates footshock-induced reinstatement of cocaine seeking (Capriles et al., 2003). There is also evidence that, as a consequence of a history of cocaine self-administration, footshock-induced CRF transmission in VTA mediates local DA and glutamate release in the region, and that these effects contribute to the circuitry mediating footshock-induced reinstatement of cocaine seeking (Wang et al., 2005; Wang et al., 2007). Although activation of CRF receptors in VTA, and of D₁-like, but not D₂-like, receptors in PFC contributes to footshock-induced reinstatement of cocaine seeking, the role of DA receptors in reinstatement induced by central injections of CRF has not been determined. Thus, the experiments presented in this chapter were designed to explore whether CRF-induced reinstatement of cocaine seeking is altered by pharmacological manipulation of D₁-like and/or D₂-like receptors. The working hypothesis, based on the neuroanatomical model presented in Chapter 1, was that i.c.v. CRF stimulates DA transmission in VTA which, in turn, activates D₁like receptors in PFC to mediate CRF-induced reinstatement of cocaine seeking. A series of four experiments (Experiments 6-9) were conducted to test this prediction.

In Experiment 6, animals with a history of cocaine self-administration were, at the time of testing for reinstatement, given systemic (i.p.) injections of the selective D_1 -like receptor antagonist, SCH23390, prior to central injections of CRF. Although previous studies have shown that systemic pretreatment with SCH23390 does not interfere in footshock-induced reinstatement of heroin seeking (Shaham and Stewart, 1996), local administration of the

antagonist in the prelimbic and orbital mPFC is effective in blocking footshock-induced reinstatement of cocaine seeking (Capriles et al., 2003). Similar results have been shown using the CPP reinstatement procedure, where antagonism of D₁-like receptors in the mPFC blocks reinstatement of cocaine-CPP induced by immobilization stress (Sanchez et al., 2003). Thus, based on my hypothesis that the effects of DA on i.c.v. CRF-induced reinstatement of cocaine seeking occur subsequent to activation of CRF receptors, I predicted that pretreatment with SCH23390 would block CRF-induced reinstatement of cocaine seeking.

In Experiment 7, I examined whether activity at DA D₂-like receptors mediates CRFinduced reinstatement of cocaine seeing. Like D₁-like receptors, D₂-like receptors are highly expressed in terminal regions of midbrain DA systems, including the PFC (Sanchez et al., 2003; Steketee, 2003). In contrast to the effects of D₁-like receptor antagonists on stressinduced reinstatement of drug seeking, both systemic and local injections of D₂-like antagonists in mPFC fail to interfere in the footshock-induced reinstatement of heroin and cocaine seeking, respectively. Thus, I predicted here that systemic pretreatment with the D₂like receptor antagonist, raclopride, would not influence the effects of i.c.v. CRF on the reinstatement of cocaine seeking.

It is well known that, depending on dose range, the administration of D_1 - and D_2 -like receptor antagonists has the potential to compromise motor activity (Hoffman and Beninger, 1985; Amalric et al., 1986; Hillegaart and Ahlenius, 1987). These effects are not surprising given the important role DA transmission plays in motor control. Thus, to examine whether any attenuation of lever responses observed in Experiments 6 or 7 could be attributed to a general motoric effect of the antagonists, I carried out two additional experiments (Experiments 8 and 9) to test the effects of SCH23390 and raclopride, at the doses administered in the reinstatement experiments, on self-administration of sucrose pellets.

Experiment 6: Effects of the D₁-like receptor antagonist, SCH23990, on i.c.v. CRFinduced reinstatement of cocaine seeking

Materials and Methods

Subjects

Thirty-six male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals), CRF (Sigma-Aldrich) and SCH23390 (Sigma-Aldrich) were all dissolved in sterile, physiological saline. CRF was centrally administered according to the microinjection procedures described in the General Methods (Chapter 2). The vehicle used in all pretreatment and test challenge conditions in this experiment was physiological saline. The doses of SCH23390 (0.05, 0.1 mg/kg, i.p.) used in this study was selected on the basis of previous work showing effective blockade within this dose range of neurotensin-induced reinstatement of cocaine seeking (Lopak and Erb, 2005), and CRF-enhanced acoustic startle responses (Meloni et al., 2006).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days according to the procedures described in the General Methods (Chapter 2).

Phase 2: Drug Free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4), with the exception of the timing and route of sham/saline injections on Extinction Day 3. In this experiment, acclimatization procedures involved an i.p. injection of saline immediately following the first extinction session, followed 30-min later by a sham i.c.v. injection. Thirty minutes after the sham injection, the second extinction session began.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days, as described in the General Methods (see Figure 4). Briefly, at the time of testing for reinstatement, animals were given systemic injection of SCH23390 (0, 0.05, 0.1 mg/kg, i.p.) 30-min prior to central injections of CRF (0.5 μg; i.c.v). Thirty minutes following the i.c.v. injection of CRF, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with the 0, 0.05, or 0.1 mg/kg dose of SCH23390, but each animal was tested after CRF (0.5 μ g) and its vehicle, on alternate days and in a counterbalanced order.

Results

Self-administration Training

Once acquired, rats maintained a stable rate of cocaine self-administration over the course of the training period. The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 36.06 ± 2.33 and 35.18 ± 2.92 infusions in each 180-min session, corresponding to an intake of 8.09 ± 0.67 mg on the final day of training (see Figure 6).

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever, compared to the last 2 days of cocaine selfadministration (63.55 ± 14.96 , 54.11 ± 15.62 , 62.80 ± 15.61 , responses on the active lever in the first 60-min extinction session for rats subsequently assigned to the 0, 0.05, 0.1 SCH 23390 dose groups, respectively).

In the extinction sessions preceding the tests for reinstatement, the number of responses on the previously active lever was very low (extinction session before first test for reinstatement: 9.54 ± 2.45 , 9.60 ± 2.68 , and 8.62 ± 2.07 responses in 60-min for rats in the 0, 0.05 and 0.1 mg/kg dose groups, respectively; extinction session before second test of reinstatement: 8.93 ± 2.34 , 6.67 ± 1.92 , and 9.92 ± 2.31 responses in 60-min for rats in the 0, 0.05 and 0.1 mg/kg dose groups, respectively; see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 16 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals had been pretreated with SCH23390 (0, 0.05, 0.1 mg/kg) and challenged with i.c.v. injections of VEH or CRF (0.5 µg) prior to testing. It can be seen in Figure 16A that CRF robustly reinstated responding on the previously active lever, and pretreatment with the higher dose of SCH23390 (0.1 mg/kg) attenuated this effect. A repeated measures ANOVA confirmed these observations, revealing a significant main effect of Test Challenge (*F*[1, 33]=39.06, *p*<0.001), and interaction of Pretreatment by Test Challenge (*F*[2,33]=4.54, *p*<0.02). Separate paired samples *t*-tests for each pretreatment condition revealed a significantly higher level of responding in the CRF relative to VEH condition in animals pretreated with the 0 mg/kg (*t*[12]=3.54, *p*<0.01) and 0.05 mg/kg (*t*[11]=6.13, *p*<0.001) dose of SCH23390, but not the 0.1 mg/kg (*p*>0.1) dose.

As can be seen in Figure 16B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,34]=9.59, p<0.01), reflecting slightly higher responding following i.c.v. CRF than VEH, there was no interaction of Pretreatment by Test Condition (p>0.6).

Experiment 7: Effects of the D₂-like receptor antagonist, raclopride, on CRF-induced reinstatement of cocaine seeking

Materials and Methods

Subjects

Twenty-eight male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Figure 16



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with SCH23390 (0 [*n*=13], 0.05 [*n*=12], 0.1 mg/kg [*n*=11]; i.p.) and challenged with VEH or CRF (0.5 µg; i.c.v.) prior to testing.

* CRF different from VEH test challenge, p < 0.01.
Surgery

Animals were surgically prepared with both an i.v. catheter and i.c.v. cannula using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals), CRF (Sigma-Aldrich) and raclopride (Sigma-Aldrich) were all dissolved in sterile, physiological saline. CRF was centrally administered according to the microinjection procedures described in the General Methods (Chapter 2). The vehicle used in all pretreatment and test challenge conditions in this experiment was physiological saline. Like SCH23390, the doses of raclopride (0.5, 0.25 mg/kg, i.p.) used in this study were selected based on previous studies carried out in the Erb laboratory (Lopak and Erb, 2005).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2, also refer to Figure 4), with the exception of the timing and route of sham/saline injections on Extinction Day 3. In this experiment, acclimatization procedures involved an i.p. injection of saline immediately following the first extinction session, followed 30-min later by a sham i.c.v. injection. Thirty minutes after the sham injection, the second extinction session began.

Phase 4: Tests for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase (see Figure 4). Subsequently, animals were given systemic injection of raclopride (0.25, 0.5 mg/kg, i.p.) 30-min prior to central injections of CRF ($0.5 \mu g$; i.c.v). Thirty minutes following the i.c.v. injection of CRF, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with the 0, 0.25, or 0.5 mg/kg dose of raclopride, and each animal was tested with CRF ($0.5 \mu g$) and its vehicle on alternate days, in a counterbalanced order.

Results

Self-administration Training

Once animals acquired cocaine self-administration, they maintained a stable rate of self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 35.69 \pm 2.10 and 34.64 \pm 2.49 infusions in each 180-min session, corresponding to an intake of 7.97 \pm 0.57 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever, compared to the last 2 days of cocaine selfadministration (50.50 ± 13.57 , 47.88 ± 10.23 , 68.72 ± 15.13 responses on the active lever in the first 60-min extinction session for rats subsequently assigned to the 0, 0.25 and 0.5 mg/kg raclopride dose groups, respectively).

In the extinction sessions preceding the tests for reinstatement, the number or responses on the previously active lever was very low (extinction session before first test for reinstatement: 9.94 ± 3.93 , 8.47 ± 2.09 , 8.84 ± 1.60 responses in 60-min for rats in the 0, 0.25 and 0.5 mg/kg raclopride dose groups, respectively; extinction session before the second test for reinstatement: 9.06 ± 2.22 , 8.50 ± 1.81 , 6.68 ± 1.16 responses in 60-min for rats in the 0, 0.25 and 0.5 mg/kg raclopride dose groups, respectively; see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 17 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals were pretreated with raclopride (0, 0.25, 0.5 mg/kg) and challenged with i.c.v. injections of VEH or CRF (0.5 µg) prior to testing. CRF reinstated responding on the active lever, as revealed by a significant main effect of Test Challenge (F[1, 25]=12.11, p<0.01). Although, in contrast to what was observed in Experiment 6, the interaction of Pretreatment by Test Challenge was in this case non-significant (F[2,25]=2.35, p>0.1), visual inspection of Figure 17A would suggest that the higher dose of raclopride did in fact interfere in the effects of CRF on reinstatement. Indeed, a separate paired-samples t-test for this group, comparing responding in the CRF and VEH group, confirms this observation (0.5 mg/kg; p>0.2), whereas a similar *t*-test performed for the 0.25 dose group was significant (t[8]=2.68, p<.05). Thus, in contrast to my predictions, it would seem that, at least at the higher dose administered, raclopride did in fact interfere in CRF-induced reinstatement of cocaine seeking.

As can be seen in Figure 17B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever again revealed a significant main effect of Test Challenge (F[1,23]=8.96, p<0.001), reflecting slightly higher responding following i.c.v. CRF than its vehicle, there was no interaction of Pretreatment by Test Condition (p>0.6).

Figure 17



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with raclopride (0 [*n*=9], 0.25 [*n*=9], 0.5 mg/kg [*n*=10]; i.p.) and challenged with VEH or CRF (0.5 µg; i.c.v.) prior to testing. * CRF different from VEH test challenge, *p*<0.05.

Experiment 8: Effects of the D₁-like receptor antagonist, SCH23990 on responding for sucrose pellets

Materials and Methods

Subjects

Eleven male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Apparatus

All behavioural procedures were carried out in sucrose self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

SCH23390 was diluted and administered at the same doses previously described in Experiment 6 of this chapter.

Procedures

Training for Self-Administration of Sucrose Pellet

Animals were trained to lever press for sucrose pellets for 8-10 days, according to the procedures described in the General Methods (Chapter 2; also refer to Figure 5).

Testing for non-specific motoric effects of SCH23390

For 2-3 sessions immediately following the final training session, the effect of SCH23390 (0.05 or 0.1 mg/kg, i.p.) on sucrose pellet consumption was assessed, according to the procedures described in the General Methods (Chapter 2; also refer to Figure 5).

Results

Once animals acquired sucrose self-administration behavior, they maintained a stable rate of responding over the course of the 8-10 day training period. The mean (\pm SEM) number of reinforced responses made on the last 2 days of training was 97.9 \pm 10.3 and 103.5 \pm 11.4 sucrose pellets respectively, in each 180-min session.

Figure 18 shows the mean (\pm SEM) total number of reinforced responses on the active (A) and inactive (B) lever during the 180-min tests for sucrose self-administration following the administration of SCH23390 (0, 0.05 or 0.1 mg/kg). It can be seen that pretreatment with the different doses of SCH23390 were without effect on active lever responding for sucrose pellets throughout the 180-min session (p's > 0.5). Responding on the inactive lever was also unaffected by pretreatment with SCH23390 (p's > 0.1; see Figure 18B).

Experiment 9: Effects of the D₂-like receptor antagonist, raclopride on responding for sucrose pellets

Materials and Methods

Subjects

Ten male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Apparatus

All behavioural procedures were carried out in sucrose self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Figure 18



Mean (\pm SEM) total number of reinforced responses (A) and inactive levers responses (B) during the 180-min tests for sucrose self-administration following the administration of SCH23390 (0 [*n*=11], 0.05 [*n*=11], 0.1 mg/kg [*n*=11]; i.p.).

Drugs

Raclopride was diluted and administered at the same doses previously described in Experiment 7 of this chapter.

Procedures

Training for Self-administration of Sucrose Pellets

Animals were trained to lever press for sucrose pellets for 8-10 days using the procedures described in the Sucrose Self-Administration section of the General Methods

Testing for Non-specific Motoric Effects of Raclopride

For 2-3 sessions immediately following the final training session, the effect of raclopride (0.25 or 0.5 mg/kg, i.p.) on sucrose pellet consumption was assessed, according to the procedures described in the General Methods (Chapter 2; also refer to Figure 5).

Results

Once animals acquired sucrose self-administration behavior, they maintained a stable rate of responding over the course of the 8-10 day training period. The mean \pm SEM number of rewarded responses made on the last 2 days of training was 99.8 \pm 8.6 and 101.2 \pm 8.2 sucrose pellets respectively, in each 180-min session.

Figure 19 shows the mean (\pm SEM) total number of rewarded responses on the active (A) and inactive (B) lever during the 180-min tests for sucrose self-administration following the administration of raclopride (0, 0.25 or 0.5 mg/kg). Consistent with the effects of the D₁-like antagonist tested in Experiment 8, it can be seen that pretreatment with the different doses

Figure 19



Mean (\pm SEM) total number of reinforced responses (A) and inactive levers responses (B) during the 180-min tests for sucrose self-administration following the administration of raclopride (0 [*n*=10], 0.25 [*n*=10], 0.5 mg/kg [*n*=10]; i.p.).

of raclopride were without effect on responding on the active lever for sucrose pellets (*p*'s >0.1). Responding on the inactive lever was also unaffected by pretreatment with raclopride (*p*'s > 0.2; see Figure 19B).

Discussion

The results of these experiments demonstrate a role for both D_1 - and potentially D_2 like receptors in CRF-induced reinstatement of cocaine seeking; indeed, pretreatment with either SCH23390 or raclopride dose-dependently attenuated responding on the previously drug-reinforced lever during tests for CRF-induced reinstatement. The observed attenuation of responding in these experiments is unlikely due to general reductions in motoric function produced by the DA antagonists used, because pretreatment with neither SCH23390 nor raclopride, at the doses administered in the reinstatement studies, affected lever pressing for sucrose pellets.

The results of Experiment 6, showing that pretreatment with SCH23390 dosedependently blocks CRF-induced reinstatement, is consistent with previous reports demonstrating a role for D_1 -like receptors in the reinstatement of cocaine seeking by central injections of other stress-related neuropeptides, including i.c.v. injections of neurotensin (Lopak and Erb, 2005), and intra-VTA injections of a Substance P analogue (Placenza et al., 2004). Likewise, the results are also consistent with the finding that selective antagonism of D_1 -like receptors attenuates the reinstatement of extinguished heroin seeking induced by 48-h food deprivation (Tobin et al., 2009), a stressor whose effects on reinstatement are known to be mediated via CRF systems (Shalev et al., 2006). Finally, the present results are consistent with the effects of SCH23390 on the expression of another CRF-induced behaviour, namely, the

potentiation of the acoustic startle response; in this study, SCH23390 dose-dependently reduced the startle enhancing effects of CRF (Meloni et al., 2006).

A likely brain site mediating the effects of the SCH23390 on CRF-induced reinstatement of cocaine seeking is the mPFC. As mentioned, antagonism of D₁-like receptors in the prelimbic and orbital regions of the mPFC blocks footshock-induced reinstatement of cocaine seeking (Capriles et al., 2003). Likewise, antagonism of D₁-like receptors in the mPFC blocks reinstatement of cocaine-CPP induced by immobilization stress (Sanchez et al., 2003). In addition, it has been found that administration of the mixed D₁/D₂-like antagonist, fluphenazine, in the dorsal mPFC interferes in footshock-induced reinstatement of cocaine seeking (McFarland et al., 2004). Given the well-established role of CRF in stress-induced reinstatement of cocaine seeking and CPP (Erb et al., 1998; Erb et al., 2006; Wang et al., 2006), as well as evidence that i.c.v. CRF reliably induces the release of DA in the mPFC (Lavicky and Dunn, 1993), there is good reason to speculate that, in the present study, D₁-like receptors in the mPFC were at least partially responsible for the effects of SCH23390 on CRF-induced reinstatement of cocaine seeking.

In contrast to the present and previous results indicating a role for D_1 -like receptors in CRF-induced reinstatement of cocaine seeking, Shaham and Stewart (1996) reported that systemic pretreatment with SCH23390 (0, 0.05, 0.1 mg/kg) was ineffective in attenuating footshock-induced reinstatement of extinguished heroin seeking. The reason for this discrepancy is unclear given that the reinstatement procedures and dose ranges of SCH23390 used in the two studies were similar. One possibility is that the population of DAergic neurons activated by footshock may be more extensive and phenotypically diverse than that activated by CRF. For example, results from electrophysiological studies conducted on VTA DA

neurons show that only a specific subpopulation of DA neurons in the VTA increase in excitatory post-synaptic currents in response to CRF (Ungless et al., 2003).

Another possible explanation for the discrepancy between the present findings and those of Shaham and Stewart (1996) is a difference in drug history, as the earlier findings were obtained in animals with a history of heroin, rather than cocaine, self-administration. While antagonism of both D_1 - and D_2 -like receptors was required to interfere in footshock-induced reinstatement of heroin seeking (Shaham and Stewart, 1996), antagonism of either receptor population was sufficient to interfere in CRF-induced reinstatement of cocaine seeking (Experiments 6 and 7). As such, it is conceivable that cocaine self-administration induces neuroadaptations that render animals with such training more sensitive than heroin-trained animals to manipulations of DA on stress-induced reinstatement of drug seeking.

Although the collective results of Experiments 6 and 7 suggest that both D_1 - and D_2 like receptors play an important role in CRF-induced reinstatement of cocaine seeking, the results of Experiment 7 were unexpected. That is, pretreatment with raclopride was not predicted to effect CRF-induced reinstatement of cocaine seeking, given that antagonism of D_2 -like receptors has previously failed to interfere with reinstatement induced by a variety of environmental stressors (Shaham and Stewart, 1996; Tobin et al., 2009) and pharmacological manipulation of stress-related systems (Placenza et al., 2004; Lopak and Erb, 2005). However, closer inspection of earlier work warrants some speculation of a role for D_2 -like receptors in stress-induced reinstatement. For example, pretreatment with raclopride (0.05 – 0.1 mg/kg) attenuates food deprivation-induced reinstatement of heroin seeking by approximately 40-45%; however, this effect was not statistically significant (Tobin et al., 2009). Moreover, pretreatment with the mixed D_1/D_2 receptor antagonist, flupenthixol, but neither D_1 - nor D_2 like antagonists alone, interfered in footshock-induced reinstatement of heroin seeking

(Shaham and Stewart, 1996). Therefore, these findings may, like the present results, point to a role for both receptor subtypes in stress-induced reinstatement of cocaine seeking.

Regardless of any discrepancies between the present and previous results concerning a role for D_2 -like receptors in stress-induced reinstatement, the current findings are consistent with the neuroanatomical model presented in Chapter 1, suggesting that downstream activation of DA receptors by CRF mediates stress-induced reinstatement of cocaine seeking. Although the present findings do not permit conclusions to be made about the loci of CRF-DA interactions in the reinstatement of cocaine seeking, sound speculation can be made based on what is known about their sites of convergence, and the neuroanatomical pathways involved in the effects of DA and CRF on the reinstatement of drug seeking. More specifically, I argue here that i.c.v. injections of CRF act to stimulate DA transmission in the VTA, most likely indirectly via an interaction with glutamate (Wang et al., 2005), to in turn facilitate DA release and postsynaptic activation of DA receptors in mPFC.

Activity of midbrain glutamatergic systems warrants further mention as this system likely plays a crucial role in the CRF-DA interaction that mediates stress-induced reinstatement. Although the actions of this transmitter were not addressed in the present series of experiments, electrophysiology and microdialysis studies show that the ability of CRF to modulate the activity of DAergic neurons requires glutamatergic mechanisms. For example, the work of Wang et al (2005) demonstrates that in cocaine experienced animals, footshockinduced release of CRF in the VTA acquires control over local glutamate release, which subsequently stimulates local DA release in the VTA. Likewise, Ungless and colleagues (2003) showed that excitatory postsynaptic currents (EPSCs) recorded in midbrain DA neuron slices, were mediated by NMDA-receptors, and that these EPSCs were potentiated by the application of CRF in a concentration-dependent manner. Recently, these results have been

extended to demonstrate that repeated cocaine exposure enhances the CRF-induced potentiation of NMDA-receptor EPSCs in VTA DA neurons, compared to that seen in naïve or saline pretreated mice (Hahn et al., 2009). In addition, application of CRF leads to a small but significant changes in potential in VTA 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA)-receptor mediated EPSCs in cocaine-treated, but not naïve or saline pretreated mice (Hahn et al., 2009). Taken together, these results strongly suggest that the functional interaction between CRF and DA demonstrated in the present experiments is likely to be mediated indirectly via glutamate receptors and transmission.

CHAPTER 5

The Role of NA and DA in Yohimbine-Induced Reinstatement of

Cocaine Seeking

Chapter 5: The Role of NA and DA in Yohimbine-induced Reinstatement of

Cocaine Seeking

Yohimbine, described as a prototypical α_2 -adrenoceptor antagonist, is widely applied in the neuropharmacological research of stress, anxiety and memory processes. More recently, yohimbine has been used as a pharmacological stressor to induce the reinstatement of drug (Lee et al., 2004; Shepard et al., 2004; Le et al., 2005; Fletcher et al., 2008; Chauvet et al., 2009; Kupferschmidt et al., 2009), and food seeking (Ghitza et al., 2006; Nair et al., 2009; Nair et al., 2011), in addition to the reinstatement of drug-induced CPP (Mantsch et al., 2010). The ability of yohimbine to induce reinstatement in both rats and non-human primates has been thought to be dependent on α_2 -adrenoceptor mediated mechanisms, based on reports that pharmacological suppression of NA transmission with α_2 -adrenoceptor agonists attenuates vohimbine-induced reinstatement of alcohol seeking in rats (Le et al., 2005), as well as reinstatement of cocaine seeking in non-human primates (Lee et al., 2004). There is mounting evidence, however, that yohimbine may in fact induce reinstatement of drug seeking via mechanisms other than, or in addition to, NA. For example, pretreatment with either the 5-HT_{2C} agonist, Ro60-0175, or 5-HT_{1A} antagonist, WAY100,635, attenuates yohimbine-induced reinstatement of cocaine and alcohol seeking, respectively (Fletcher et al., 2008; Dzung Le et al., 2009). In addition, recent studies by Nair and colleagues (Nair et al., 2010; Nair et al., 2011) demonstrated that pretreatment with the D_1 -like antagonist, SCH23390, or D_2 -like antagonist, raclopride, blocked vohimbine-induced reinstatement of high-fat food seeking, suggesting a role for DA in this reinstatement.

The series of experiments presented in this chapter represent an exploration of the neuropharmacology of yohimbine-induced reinstatement of cocaine seeking, focusing on the roles of α_2 -adrenoceptor, D₁, and D₂-like receptors. The rationale for exploring a role for DA

was based on knowledge that yohimbine acts to enhance DA release in brain regions known to be involved in stress-induced reinstatement, such as the PFC (Millan et al., 2000). Moreover, I have recent evidence to show that systemic injection of yohimbine selectively increases extracellular levels of DA in the mPFC of cocaine, but not saline, pre-exposed animals (see Appendix A). This finding suggests that a history of cocaine self-administration may enhance DA transmission in the mPFC, a region known to be important in the effects of stress on reinstatement of drug seeking.

Experiment 10 addressed the hypothesis that α_2 -adrenoceptors are responsible for the effects of yohimbine on the reinstatement of cocaine seeking. In this experiment, animals were pretreated with the α_2 -adrenoceptor agonist, clonidine, at doses known to inhibit NA transmission and release, prior to tests for yohimbine-induced reinstatement. Although clonidine has been found to significantly attenuate yohimbine-induced reinstatement of cocaine seeking in monkeys (Lee et al., 2004) and alcohol seeking in rats (Dzung Le et al., 2009), other studies have failed to show an effect of clonidine pretreatment on yohimbine-induced reinstatement of food seeking (Nair et al., 2009; Nair et al., 2010) or yohimbine-induced reinstatement of a cocaine-CPP in mice (Mantsch et al., 2010). Moreover, selective 6-OHDA lesions of the dorsal or ventral NAergic pathway fails to attenuate yohimbine-induced reinstatement of alcohol seeking in rats (Dzung Le et al., 2009).

After obtaining a null effect of clonidine on yohimbine-induced reinstatement of cocaine seeking in Experiment 10, Experiments 11 and 12 examined whether DAergic signaling via D₁-like receptors mediates yohimbine-induced reinstatement of cocaine seeking. As previously mentioned, Nair and colleagues (Nair et al., 2010; Nair et al., 2011) reported that both systemic and intra-dorsal mPFC injections of SCH23390, blocked yohimbine-induced reinstatement of high-fat food seeking.

found to interfere in other forms of stress-induced reinstatement of cocaine seeking, including by footshock (when injected into the PFC; Capriles et al., 2003), intra-VTA injections of Substance P (Placenza et al., 2004), i.c.v. injections of neurotensin (Lopak and Erb, 2005) and i.c.v. injections of CRF (Experiment 6, Chapter 4). Based on these findings, I predicted that pretreatment with SCH23390 would block yohimbine-induced reinstatement.

Although SCH23390 is a potent and selective blocker of D₁-like receptors (Iorio et al., 1983), it has been found in a certain dose range (>1.5 mg/kg) to also interact with 5-HT₂ receptors in some brain regions, including the PFC (Bischoff et al., 1986; McQuade et al., 1988). As previously mentioned 5-HT_{1A} and 5-HT_{2C} receptors have been implicated in yohimbine-induced reinstatement of alcohol and cocaine seeking, respectively (Fletcher et al., 2008; Dzung Le et al., 2009). Thus, to confirm that any effects of SCH23390 observed in Experiment 11 were independent of 5-HT mechanisms, I conducted an additional study (Experiment 12) using the more selective D_1 -like receptor antagonist, SCH31966, which has significantly lower affinity than SCH23390 for 5-HT₂ (McQuade et al., 1988; Wamsley et al., 1991) and 5HT_{1C} (Taylor et al., 1991; Wamsley et al., 1991) receptors. Subsequently, Experiment 13 was carried out to confirm that the dose of SCH31966 administered in Experiment 12, did not produce generalized motoric effects. This was accomplished by assessing the effects of the antagonist on sucrose self-administration behaviour, as was conducted for SCH23390 and raclopride in the previous Chapter (Experiments 8 and 9, Chapter 4).

Finally, in Experiment 14, animals with a history of cocaine self-administration were, at the time of testing for reinstatement, given systemic injections of the selective D_2 -like receptor antagonist, raclopride, prior to systemic injection of yohimbine. As previously mentioned, yohimbine acts as a D_2 -like receptor antagonist itself, inhibiting autoreceptors on ascending

DAergic neurons (Gobert et al., 1995; Gobert et al., 1998). Accordingly, administration of yohimbine markedly increases striatal synthesis of DA (Scatton et al., 1980) and induces DA release in the PFC (Millan et al., 2000; Appendix A). The effects of raclopride on midbrain DA systems are in fact similar to that of yohimbine. Indeed, following administration of raclopride, DA levels also increase in the NAc and PFC (See et al., 1991; Gobert et al., 1998), although the increase induced by yohimbine tend to be considerably higher than that induced by raclopride. From this perspective, it is of interest that raclopride, itself, does not induce reinstatement (Experiments 9,15; Shaham et al., 1996). Likewise, systemic injections of raclopride have failed to interfere in reinstatement of drug seeking by other forms of stress, including footshock (Capriles et al., 2003), intra-VTA injections of Substance P (Placenza et al., 2004), and i.c.v. injections of neurotensin (Lopak and Erb, 2005). Taken together, these findings support a hypothesis that raclopride should not interfere in the yohimbine-induced reinstatement of cocaine seeking.

Experiment 10: Effects of a2-adrenoceptor agonist Clonidine on yohimbine-induced reinstatement of cocaine Seeking

Material and Methods

Subjects

Thirty-six male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with an i.v. catheter using the procedures described in the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and clonidine (Sigma-Aldrich) were dissolved in sterile, physiological saline. Yohimbine (Sigma-Aldrich) was dissolved in distilled water. Clonidine and yohimbine were administered systemically (i.p.) following the same procedures and doses used in Experiments 3 and 4 (Chapter 3).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine for 8-10 days, according to the procedures described in the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4). Injection acclimatization procedures on Day 3 included an i.p. injection of saline immediately after the first extinction session, followed 30 min later by an i.p.

injection of distilled water. Thirty minutes after the second saline injection, Extinction Session 2 began.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days, as described in the General Methods (see Figure 4). Briefly, at the time of testing for reinstatement, animals were given systemic injections of clonidine (0, 40 μ g /kg, i.p.) 30 min prior to systemic injections of yohimbine (0 and 0.625 mg/kg or 0 and 1.25 mg/kg; i.p.). Thirty minutes following the i.p. injection of yohimbine, tests for reinstatement were given, whereby the previously drug-reinforced lever was presented and responding was recorded for 60-min. Different groups of animals were pretreated with the 0 or 40 μ g /kg dose of clonidine, but each animal was tested after the 0 mg/kg dose of yohimbine and either the 0.625 or 1.25 mg/kg dose of yohimbine, on alternate days in a counterbalanced order.

Results

Because animals in the 0.625 and 1.25 mg/kg yohimbine dose conditions were trained and tested in independent groups, the data were analyzed separately.

Self-administration Training

Once cocaine self-administration was acquired, rats maintained a stable intake over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was 32.61 ± 1.54 and 33.03 ± 1.68 infusions in each 180-min session for rats in the, respectively. This training data corresponded to an average intake of 7.59 ± 0.38 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever, compared to the last 2 days of cocaine selfadministration. In the 0.625 mg/kg yohimbine dose condition, the clonidine pretreatment group averaged 83.80 ± 16.37 and the vehicle pretreatment group averaged 77.62 ± 9.31 responses in the first 60-min extinction session (i.e., Extinction Day 1, session 1). Responding rates for animals assigned to the clonidine pretreatment group, gradually declined to a low 10.73 ± 2.97 and 12.91 ± 3.61 in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Similarly, animals assigned to the vehicle pretreatment group declined to a low 6.12 ± 2.50 and 8.57 ± 3.87 in the extinction session immediately prior to reinstatement test 1 and 2, respectively.

In the 1.25 mg/kg yohimbine dose condition, the clonidine pretreatment group averaged 114.75 ± 16.06 and the vehicle pretreatment group averaged 92.67 ± 19.77 responses in the first 60-min extinction session (i.e., Extinction Day 1, session 1). Responding rates for animals assigned to the clonidine pretreatment group, gradually declined to a low 11.33 ± 2.69 and 9.67 ± 43.34 in the extinction session immediately prior to reinstatement test 1 and 2, respectively. Similarly, animals assigned to the vehicle pretreatment group declined to a low 10.67 ± 4.06 and 6.33 ± 2.56 in the extinction session immediately prior to reinstatement test 1 and 2, respectively (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 20 and 21 show the mean (<u>+</u>SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated

with clonidine (0, 40 µg/kg) and challenged with the 0.625 or 1.25 mg/kg dose of yohimbine, respectively, prior to testing. As can be seen in Figures 18A and 19A, yohimbine reinstated responding on the previously active lever, relative to responding induced by VEH, and pretreatment with clonidine failed to attenuate this effect. Consistent with these observations, a repeated measures ANOVA revealed only main effects of Test Challenge (*F*[1,16]=10.18, p<0.01 and *F*[1,15]=4.73, p<0.05 for the 0.625 and 1.25 mg/kg doses, respectively); there were no effects of clonidine pretreatment (p>0.6), nor did these factors interact.

As can be seen in Figures 20B and 21B, responding on the inactive lever was very low (less than 5 responses) across all pretreatment and test challenge conditions. Although repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,16]=5.92, p<0.03 and F[1,16]=5.92, p<0.03 for the 0.625 and 1.25 mg/kg doses, respectively), reflecting slightly higher responding following i.c.v. NA than its vehicle, there was no interaction of Pretreatment by Test Condition (p>0.1).

Experiment 11: Effects of the D₁-like receptor antagonist, SCH23990, on yohimbineinduced reinstatement of cocaine seeking

Materials and Methods

Subjects

Thirty-three male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with an i.v. catheter using the procedures described in Surgery section of the General Methods (Chapter 2).

Figure 20



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with clonidine (0 [*n*=7], 40 µg/kg [*n*=11]; i.p.) and challenged with VEH or yohimbine (0.625 mg/kg; i.p.) prior to testing. * Yohimbine (0.625 mg/kg) different from VEH test challenge, *p* <0.05.

Figure 21



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with clonidine (0 [*n*=6], 40 µg/kg [*n*=12]; i.p.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing. * Yohimbine (1.25 mg/kg) different from VEH test challenge, *p* <0.01.

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and SCH23390 (Sigma-Aldrich) were dissolved in sterile, physiological saline. Yohimbine (Sigma-Aldrich) was dissolved in distilled water. Both yohimbine and SCH23390 were administered systemically (i.p.) at doses established in Experiments 4, 6 and 8 (Chapters 3 and 4). The vehicle administered in the pretreatment condition was saline, whereas the vehicle administered in the test challenge condition was distilled water.

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days according to the procedures described in the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4), with the exception of the specifics of the timing of saline

acclimatization injections on Extinction Day 3. In this experiment, acclimatization involved an i.p. injection of saline immediately after the first extinction session, followed 30-min later by an i.p. injection of distilled water. Thirty minutes after the second saline injection, Extinction Session 2 commenced.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 3 consecutive days, as described in the General Methods (see Figure 4). Briefly, at the time of testing for reinstatement, animals were given systemic injection of SCH23390 (0, 0.05, 0.1 mg/kg, i.p.) 30-min prior to systemic injections of yohimbine (1.25 mg/kg, i.p.). Thirty minutes following administration of yohimbine, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with the 0, 0.05, or 0.1 mg/kg dose of SCH23390, but each animal was tested after yohimbine and its vehicle, on alternate days and in a counterbalanced order.

Results

Self-administration Training

Once acquired, rats maintained a stable rate of cocaine self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 34.3 ± 4.6 and 29.9 ± 3.5 infusions in each 180-min session, corresponding to an intake of 6.88 ± 0.84 mg on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding (compared to the last 2 days of cocaine self-administration) on the previously drugreinforced lever averaging 62.6 ± 18.7 , 43.9 ± 8.2 , and 63.3 ± 15.6 responses on the active lever in the first 60 min extinction session for rats subsequently assigned to the 0, 0.05, 0.1 SCH23390 dose groups, respectively.

In the extinction sessions just preceding the tests for reinstatement, number of responses on the previously active lever was very low (extinction session before first test of reinstatement: 6.1 ± 1.0 , 15.4 ± 5.1 , and 6.8 ± 2.1 responses in 1-h for rats in the 0, 0.05, 0.1 SCH23390 dose groups, respectively; extinction session before second test of reinstatement: 1.6 ± 1.0 , 1.4 ± 0.5 , and 2.0 ± 1.4 responses in 60 min for rats in the 0, 0.05, 0.1 SCH23390 dose groups (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 22 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals were pretreated with SCH23390 (0, 0.05, 0.1 mg/kg) and challenged with VEH or yohimbine (1.25 mg/kg) prior to testing. It can be seen in Figure 22A that yohimbine robustly reinstated responding on the previously active lever, and that pretreatment with SCH23390 attenuated this effect. A repeated measures ANOVA confirmed these observations, revealing a significant main effect of Test Challenge (F[1,26]=12.47, p<0.001), and interaction of Pretreatment by Test Challenge (F[2,26]=3.53, p<0.05). Separate paired samples *t*-tests for each pretreatment condition

Figure 22



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with SCH23390 (0 [n=10], 0.05 [n=11], 0.1 mg/kg [n=10]; i.p.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing.

* Yohimbine different from VEH test challenge, p < 0.05.

revealed a significantly higher level of responding in the yohimbine relative to VEH condition in animals pretreated with 0 and 0.05 mg/kg SCH23390 (t[8]=2.38, p<0.05, t[9]=2.88, p<0.02), but not in rats pretreated with 0.1 mg/kg SCH23390 (p>0.05).

As can be seen in Figure 22B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,28]=7.13, p<0.02), reflecting slightly higher responding following yohimbine than VEH, there was no interaction of Pretreatment by Test Condition (p>0.1).

Experiment 12: Effects of the D₁-like receptor antagonist, SCH31966, on yohimbineinduced reinstatement of cocaine seeking

Materials and Methods

Subjects

Sixteen male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Surgery

Animals were surgically prepared with an i.v. catheter using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and SCH39166 (Sigma-Aldrich) were dissolved in sterile, physiological saline. Yohimbine (Sigma-Aldrich) was dissolved in distilled water. The vehicle administered in the pretreatment condition was saline, whereas the vehicle administered in the test challenge condition was distilled water. The dose of SCH31966 (0.2 mg/kg) was chosen based on the effects of the antagonist on locomotor activity, cocaine- and liquid food self-administration (Barrett et al., 2004; Collins et al., 2010). Yohimbine was administered systemically (i.p.) using the procedures and dose established in Experiment 4 (Chapters 3).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days according to the procedures described in the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction Days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4), with the exception of the specifics of the timing of saline acclimatization injections on Extinction Day 3. In this experiment, acclimatization procedures involved an i.p. injection of saline immediately after the first extinction session, followed 30-

min later by an i.p. injection of distilled water. Thirty minutes after the second saline injection, Extinction Session 2 commenced.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 2 consecutive days, as described in the General Methods (see Figure 4). At the time of testing for reinstatement, animals were given systemic injection of SCH31966 (0, 0.2 mg/kg, i.p.) 30-min prior to systemic injection of yohimbine (0, 1.25 mg/kg, i.p.). Thirty minutes following administration of yohimbine, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with 0 or 0.2 mg/kg SCH31966, but each animal was tested after VEH or yohimbine (1.25 mg/kg), on alternate days and in a counterbalanced order.

Results

Self-administration Training

Once acquired, rats maintained a stable rate of cocaine self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 33.94 \pm 2.58 and 31.31 \pm 2.82 infusions in each 3-h session, corresponding to an intake (mg) of 7.20 \pm 0.65 on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever, averaging 83.82 ± 15.29 and 90.61 ± 10.48

responses on the active lever in the first 60-min extinction session for rats subsequently assigned to the 0, 0.2 SCH31966 dose groups, respectively.

In the extinction sessions just preceding the tests for reinstatement, number of responses on the previously active lever was very low (extinction session before first test of reinstatement: 6.82 ± 4.17 and 5.82 ± 2.24 responses in 60 min for rats in the 0, 0.2 SCH31966 dose groups dose groups, respectively; extinction session before second test of reinstatement: 6.45 ± 1.81 and 11.18 ± 2.45 responses in 60 min for rats in the 0, 0.2 SCH31966 dose groups (see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 23 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals had been pretreated with SCH31966 (0, 0.2 mg/kg) and challenged with VEH or yohimbine (1.25 mg/kg) prior to testing. It can be seen in Figure 23A that yohimbine reinstated responding on the previously active lever, and that pretreatment with SCH31966 (0.2 mg/kg) attenuated this effect. A repeated measures ANOVA confirmed these observations, revealing a significant main effect of Test Challenge (*F*[1, 14]=9.79, *p*<0.001), and interaction of Pretreatment by Test Challenge (*F*[1,14]=7.65, *p*<0.02). Separate paired samples *t*-tests for each pretreatment condition revealed a significantly higher level of responding in the yohimbine relative to VEH condition in animals pretreated with 0 mg/kg SCH31966 (*t*[7]=2.96, *p*<0.05), but not in rats pretreated with 0.2 mg/kg SCH31966 (*p*>0.1).

As can be seen in Figure 23B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for

Figure 23



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with SCH31966 (0 [n=8], 0.2 mg/kg [n=8]; i.p.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing.

* Different from all other conditions, p < 0.05.

responses on this lever revealed a significant main effect of Test Challenge (F[1,14]=5.93, p<0.05), reflecting slightly higher responding following yohimbine than VEH, there was no interaction of Pretreatment by Test Condition (p>0.1).

Experiment 13: Effects of the D₁-like receptor antagonist, SCH31966 on responding for sucrose pellets

Materials and Methods

Subjects

Ten male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Apparatus

All behavioural procedures were carried out in sucrose self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

SCH31966 was diluted and administered at the same doses described in the previous experiment (Experiment 12).

Procedures

Training for Self-administration of Sucrose Pellets

Animals were trained to lever press for sucrose pellets for 8-10 days, according to the procedures described in the General Methods (Chapter 2).
Testing for Non-specific Motoric Effects of SCH31966

For 2-3 sessions immediately following the final training session, the effect of pretreatment injections of SCH31966 (0.2 mg/kg, i.p.) was assessed, according to the procedures described in the General Methods (Chapter 2; also refer to Figure 5).

Results

Once animals acquired sucrose self-administration behavior, they maintained a stable rate of responding over the course of the 8-10 day training period. The mean \pm SEM number of rewarded responses made on the last 2 days of training was 109.45 \pm 26.89 and 114.82 \pm 25.27 pellets, respectively, in each 3-h session.

Figure 24 shows the mean (\pm SEM) total number of rewarded responses on the active (A) and inactive (B) levers during the 180-min tests for sucrose self-administration following the administration of SCH31966 (0, 0.2 mg/kg). As can be seen in the graph, pretreatment with either dose of SCH31966 was without effect on responding on the active lever for sucrose pellets throughout the 180-min session (p > 0.08). Responding on the inactive lever was also unaffected by pretreatment with SCH31966 (p > 0.1).

Experiment 14: Effects of the D₂-like receptor antagonist, raclopride, on yohimbine-induced reinstatement of cocaine seeking

Materials and Methods

Subjects

Thirty male Long Evans rats (275-300g) were housed and maintained under conditions described in the General Methods (Chapter 2), were used in this study.

Figure 24



Mean (\pm SEM) total number of reinforced responses (A) and inactive lever responses (B) during the 180-min tests for sucrose self-administration following the administration of SCH31966 (0 [n=10], 0.2 mg/kg [n=10]; i.p.).

Surgery

Animals were surgically prepared with an i.v. catheter using the procedures described in Surgery section of the General Methods (Chapter 2).

Apparatus

All behavioural procedures were carried out in drug self-administration chambers (Med Associates) described in the Apparatus section of the General Methods (Chapter 2).

Drugs

Cocaine HCl (Medisca Pharmaceuticals) and raclopride (Sigma-Aldrich) were dissolved in sterile, physiological saline. Yohimbine (Sigma-Aldrich) was dissolved in distilled water. The vehicle administered in the pretreatment condition was saline, whereas the vehicle administered in the test challenge condition was distilled water. Both yohimbine and raclopride were administered systemically (i.p.) at doses established in Experiment 4, 7 and 9 (Chapters 3 and 4).

Reinstatement Procedures

Phase 1: Cocaine Self-administration Training

Animals were trained to self-administer cocaine (0.23 mg/65 μ l infusion, i.v.) for 8-10 days using the procedures described in Phase 1 of the Reinstatement of Drug Seeking section of the General Methods (Chapter 2).

Phase 2: Drug-free Period

Animals were kept in their homecages for 7 days and maintained under vivarium conditions described in the General Methods (Chapter 2).

Phase 3: Extinction Training

Extinction days 1-3 were carried out as described in the General Methods (Chapter 2; also refer to Figure 4), with the exception of the specifics of the timing of saline acclimatization injections on Extinction Day 3. In this experiment, acclimatization procedures involved an i.p. injection of saline immediately after the first extinction session, followed 30-min later by an i.p. injection of distilled water. Thirty minutes after the second saline injection, Extinction Session 2 commenced.

Phase 4: Testing for Reinstatement

Following extinction, animals were tested for reinstatement over 3 consecutive days. Test days began with extinction sessions according to procedures employed during the extinction phase (see Figure 4). Subsequently, animals were given systemic injection of raclopride (0, 0.25, 0.5 mg/kg, i.p.) 30-min prior to systemic injections of yohimbine (0, 1.25 mg/kg, i.p.). Thirty minutes following administration of yohimbine, tests for reinstatement began, whereby the previously drug-reinforced lever was presented and responding was recorded over 60-min. Different groups of animals were pretreated with 0, 0.25, or 0.5 mg/kg dose of raclopride, but each animal was tested after yohimbine and its vehicle, on alternate days in a counterbalanced order.

Results

Self-administration Training

Once acquired, rats maintained a stable rate of cocaine self-administration over the course of the training period (see Figure 6). The mean \pm SEM number of infusions made on the last 2 days of training was, respectively, 33.90 \pm 2.40 and 29.18 \pm 3.97 infusions in each 180-min session, corresponding to an intake (mg) of 6.71 \pm 0.91 on the final day of training.

Extinction Training

At the start of the extinction phase, animals showed characteristic heightened responding on the previously drug-reinforced lever averaging 40.82 ± 8.99 , 46.10 ± 10.40 , and 53.45 ± 12.20 responses on the active lever in the first 60-min extinction session for rats subsequently assigned to the 0, 0.25, 0.5 mg/kg raclopride dose groups, respectively.

In the extinction sessions just preceding the tests for reinstatement, the number of responses on the previously active lever was very low (extinction session before first test of reinstatement: 5.27 ± 1.61 , 9.70 ± 3.20 , and 5.82 ± 1.57 responses in 60 min for rats in the 0, 0.25, 0.5 mg/kg mg/kg dose groups, respectively; extinction session before second test of reinstatement: 2.45 ± 1.02 , 5.30 ± 2.06 , and 3.82 ± 1.88 responses in 60 min for rats in the 0, 0.25, 0.5 mg/kg dose groups; see Figure 7 for a representative extinction curve depicting the decline in responding over the extinction phase).

Testing for Reinstatement

Figure 25 shows the mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during 60-min tests for reinstatement in which animals had been pretreated with raclopride (0, 0.25, 0.5 mg/kg) and challenged with VEH or yohimbine (1.25 mg/kg) prior to

Figure 25



Mean (\pm SEM) number of responses on the active (A) and inactive (B) levers during the 60-min tests for reinstatement in which animals were pretreated with raclopride (0 [*n*=9], 0.25 [*n*=9], 0.5 mg/kg [*n*=12]; i.p.) and challenged with VEH or yohimbine (1.25 mg/kg; i.p.) prior to testing

* Yohimbine different from VEH test challenge, p < 0.01.

testing. As can be seen in the graph, pretreatment with raclopride was without effect on the reinstatement of cocaine seeking induced by yohimbine. The repeated measures ANOVA revealed only a main effect of Test Challenge (F[1,27]=23.51, p<0.001); there was no effect of raclopride pretreatment (p>0.5), nor did the factors interact.

As can be seen in Figure 25B, responding on the inactive lever was very low across all pretreatment and test challenge conditions. Although a repeated measures ANOVA for responses on this lever revealed a significant main effect of Test Challenge (F[1,22]=9.19, p<0.01), reflecting slightly higher responding following yohimbine than VEH, there was no interaction of Pretreatment by Test Condition (p>0.2).

Discussion

Three major findings emerge from this series of experiments. First, systemic injections of the α_2 -adrenoceptor agonist, clonidine, administered at doses known to interfere in NA cell firing and release, failed to interfere in yohimbine-induced reinstatement of cocaine seeking. Second, it was found that antagonism of D₁-like receptors with the selective antagonists, SCH23390 and SCH31966, attenuated yohimbine-induced reinstatement of cocaine seeking. The effects of the D₁-like antagonists were unlikely due to general reductions in motoric function given that pretreatment with the same doses of the substances that were effective in the reinstatement procedure, failed to interfere in the self-administration of sucrose pellets (Experiments 8 and 13). Finally, it was found that antagonism of D₂-like receptors did not interfere with yohimbine-induced reinstatement.

In Experiment 10, the failure of clonidine to interfere with yohimbine-induced reinstatement of cocaine seeking was consistent with recent reports of a null effect of clonidine

on yohimbine-induced reinstatement of food seeking in rats (Nair et al., 2010), and yohimbineinduced reinstatement of a cocaine-CPP in mice (Mantsch et al., 2010). Indeed, in the present study clonidine, administered at a dose known to block the effects of footshock-induced reinstatement of cocaine seeking (Erb et al., 2000), had no effect on yohimbine-induced reinstatement responding when either a standard reinstatement test dose of the drug (i.e., 1.25 mg/kg; Feltenstein and See, 2006; Fletcher et al., 2008; Ghitza et al., 2006; Kupferschmidt et al., 2009; Le et al., 2005; Shepard et al., 2004) or a dose half that (0.625 mg/kg) was administered. These findings suggest that antagonism of α_2 -adrenoceptors does not mediate the effects of yohimbine on reinstatement of cocaine seeking in rats, and is consistent with the idea that yohimbine induces reinstatement of drug seeking via a mechanism other than, or at the very least in addition to, antagonism of α_2 -adrenoceptors (Dzung Le et al., 2009; Mantsch et al., 2010; Nair et al., 2011). It is important to note, however, that evidence of α_2 adrenoceptor-mediated effects of vohimbine can be found in the reinstatement literature. For example, as previously described, pretreatment with clonidine has been found to interfere in vohimbine-induced reinstatement of alcohol seeking in rats (Dzung Le et al., 2009) and cocaine seeking in monkeys (Lee et al., 2004). Moreover, the administration of highly selective α_2 -adrenoceptor antagonists, RS79948 and BRL44408, have been found to mimic the effects of vohimbine on reinstatement of cocaine seeking in monkeys and mice, respectively (Lee et al., 2004; Mantsch et al., 2010).

Although the reasons for the discrepancies in the present and other findings are unclear, they may reflect differences in several factors (and most probably a combination of factors), including the reinstatement paradigm used, drug history of the subjects, and species tested. In particular, there is other work indicating discrepancies between monkeys and rats in the effects of NAergic manipulations on reinstatement. For example, Schmidt and Pierce (2006)

demonstrated that systemic injection of the NA transporter inhibitor, nisoxetine, did not reinstate extinguished cocaine seeking in rats. In contrast, Platt and colleagues (2007) showed that nisoxetine and another NA transporter inhibitor, talsupram, dose-dependently reinstated cocaine-seeking behavior in monkeys. These species differences in the effects of NA on the reinstatement of cocaine seeking have been attributed to differences in the organization of NA systems in the brain (Platt et al., 2007).

Regardless of the extent to which NA in fact plays a role in the drug-related behavioural effects of yohimbine, it is apparent that, systems in addition to NA are involved. From this perspective, Experiments 11, 12 and 14 were designed to address a possible role for DA in the effects of yohimbine on reinstatement of cocaine seeking. My findings that D₁-like, and not D₂-like, antagonists interfered in yohimbine-induced reinstatement of cocaine seeking are consistent with the selective effects of D₁-like receptor manipulations on other forms of stress-induced reinstatement described in the introduction of this Chapter (Capriles et al., 2003; Placenza et al., 2004; Lopak and Erb, 2005). However, they are inconsistent with the findings reported in Chapter 4, where both D₁- and D₂-like antagonists attenuated reinstatement of cocaine seeking by i.c.v. injections of CRF, and with the findings of Nair and colleagues (Nair et al., 2010; Nair et al., 2011) which demonstrate that antagonism of either DA receptor population interferes in yohimbine-induced reinstatement of food seeking.

One possibility for the discrepancy between the present null effect of raclopride on yohimbine-induced reinstatement of cocaine seeking, and the attenuating effect of raclopride on yohimbine-induced reinstatement of food seeking, reported by Nair and colleagues (Nair et al., 2010), is the use of drug versus non-drug reinforcement. Indeed, repeated exposure to cocaine has been shown to alter functioning of D₂-like receptors in the PFC (Nogueira et al., 2006; Kroener and Lavin, 2010), a region shown to be involved in yohimbine-induced

reinstatement (Nair et al., 2011; Appendix A). For example, Nogueira et al (2006) demonstrate that rats with a history of cocaine exposure show a blunted D₂-like receptor-mediated excitation of PFC neurons following VTA stimulation, relative to saline-treated rats. Thus, a history of cocaine self-administration may alter D₂-like receptor-mediated transmission in the PFC, and such alterations may account for the discrepant effects of raclopride on yohimbine-induced reinstatement of cocaine and food seeking.

As previously mentioned, Nair and colleagues (2011) recently demonstrated that antagonism of D₁-like receptors in the mPFC blocks yohimbine-induced reinstatement of food seeking. Consistent with these findings, I have recently shown that yohimbine selectively enhances extracellular levels of DA in cocaine pre-exposed animals, relative to controls. These findings, taken together with previous data showing that D₁-like receptors in the mPFC play a critical role in footshock-induced reinstatement of cocaine seeking (Capriles et al., 2003) and footshock-induced reactivation of a morphine CPP (Sanchez et al., 2003), suggest that D₁-like receptors in the mPFC may represent a common mechanism of reinstatement by different forms of stress, including yohimbine.

Although not tested in the present series of experiments, it is important to note that 5-HT has also been show to play an important role in yohimbine-induced reinstatement. As discussed previously, yohimbine acts as an agonist of 5-HT_{1A} 5-HT_{1B} and 5-HT_{1D} receptors (Scatton et al., 1980; Newman-Tancredi et al., 1998; Millan et al., 2000), and administration of the drug results in increases of 5-HT levels in the PFC (Millan et al., 2000). It has also been shown that the selective 5-HT_{1A} agonist, 8-OH-DPAT, generalizes to yohimbine in tests of drug discrimination (Winter and Rabin, 1992, 1993). In a recent reinstatement study, Dzung Le and colleagues (2009) demonstrated that pretreatment with the 5-HT_{1A} antagonist, WAY100,635, significantly reduced yohimbine-induced reinstatement of alcohol seeking.

Similarly, pretreatment with Ro60-0175 blocked yohimbine-induced reinstatement of cocaine seeking (Fletcher et al., 2008). Thus, 5-HT would seem another important neurochemical system involved in the effects of yohimbine on reinstatement of cocaine seeking.

Taken together, the results of the experiments presented in this chapter, and the work of others, clearly demonstrate that yohimbine induces reinstatement via its actions at multiple receptor sites and via several neurochemical systems, including DA and 5-HT. The conclusion that NA is not involved or, at best, only indirectly involved in the effects of yohimbine on reinstatement of cocaine seeking in fact helps to explain what was initially a puzzling finding in Experiment 4 (Chapter 3); namely, that i.c.v. injection of the CRF receptor antagonist, D-Phe, failed to interfere in the effects of yohimbine on reinstatement. Based on the neuroanatomical circuitry of stress-induced reinstatement of cocaine seeking proposed in Chapter 1, and the nature of NA-CRF interactions in reinstatement described in Chapter 3, antagonism of CRF receptors should have interfered in yohimbine-induced reinstatement of cocaine seeking, if indeed yohimbine was having its effects via changes in NA transmission. The fact, however, that D-Phe blocked NA-induced reinstatement of cocaine seeking without interfering in yohimbine-induced reinstatement offers additional support for the idea that yohimbine acts via some system other than NA, most likely DA and/or 5-HT, to have its effects on the reinstatement of cocaine seeking.

CHAPTER 6

General Discussion

Chapter 6: General Discussion

The series of experiments presented in this dissertation examined whether the stress peptide, CRF, functionally interacts with the stress-related catecholamines, NA and DA, to mediate reinstatement cocaine seeking. The present findings extend on what is currently known about the independent actions of CRF, NA and DA in stress-induced reinstatement, and show directly for the first time that pharmacological manipulations of both NA and DA receptors can alter CRF-induced reinstatement of cocaine seeking. Specifically, the present findings suggest that the activation of CRF receptors is likely to occur subsequent to, and downstream of, the sites of action of NA, whereas the activation of DA receptors likely occurs subsequent to, and downstream of the effects of CRF (see Table 1 for a summary of these findings). Finally, the present findings demonstrate that, contrary to expectations, reinstatement of cocaine seeking by the prototypical α_2 -adrenoceptor antagonist, yohimbine, is in fact not mediated by alterations in NA transmission, but is modulated by alterations in DA transmission (see Table 2 for summary of these findings).

Taken together, the results presented in Chapters 3-5 are consistent with the neuroanatomical model of stress-induced reinstatement outlined in Chapter 1 (see Figure 3). Specifically, my finding that central injections of a CRF receptor antagonist interfered in NA-induced reinstatement of cocaine seeking, but that attenuation of NA transmission failed to interfere in CRF-induced reinstatement, is consistent with the notion that NA interacts with CRF-containing cells in regions such as the CeA and BNST (Leri and Stewart, 2002), and that CRF released from these cells at distal sites, including the BNST and VTA (Erb et al., 2001; Wang et al., 2005; Rodaros et al., 2007), initiates the behaviours involved in relapse. Furthermore, my findings that antagonism of D_1 - or D_2 -like receptors attenuates CRF-induced reinstatement of cocaine seeking are consistent with the notion that CRF facilitates DA

Table 1

Summary of findings from experiments examining which catecholamine systems interact with CRF to mediate the reinstatement of cocaine seeking in rats (Chapters 3 and 4)

Experiment	Direction of Interaction	Pretreatment	Stressor	Results
2	NA → CRF	D-Phe	NA	Blockade of reinstatement
3	CRF → NA	Clonidine	CRF	Reinstatement of cocaine seeking
4	NA → CRF	D-Phe	Yohimbine	Reinstatement of cocaine seeking
6	CRF \rightarrow D ₁ -like receptors	SCH23390	CRF	Dose dependent attenuation of reinstatement
7	CRF \rightarrow D ₂ -like receptors	Raclopride	CRF	Dose dependent attenuation of reinstatement

Table 2

Summary of findings from experiments examining which catecholamine systems mediate yohimbine-induced reinstatement of cocaine seeking in rats (Chapter 5)

Experiment	Pretreatment	Stressor	Results
10	Clonidine	Yohimbine	Reinstatement of cocaine seeking
11	SCH23390	Yohimbine	Dose dependent attenuation of reinstatement
12	SCH31966	Yohimbine	Blockade of reinstatement
14	Raclopride	Yohimbine	Reinstatement of cocaine seeking

transmission in the midbrain (most likely via an interaction with glutamate; Wang et al., 2005), and that blockade of DA receptors at distal sites, including the PFC, attenuates reinstatement (Capriles et al., 2003; McFarland et al., 2004). Importantly, although largely confirmatory of an existing body of literature and previously described neuroanatomical model of reinstatement, the present findings extend this work in an important way by providing the first direct evidence that simultaneous manipulation of CRF and catecholamine systems alters reinstatement of cocaine seeking in a predictable manner.

It is important to note that the neuroanatomical model outlined in Figure 3 is based on the results from localization studies that used, almost exclusively, intermittent footshock stress to trigger reinstatement. In contrast, with the exception of the vohimbine studies, the primary methodological approach used in this thesis involved the targeted pharmacological manipulation of a specific population or subpopulation of receptors to induce reinstatement. These manipulations, including central activation of NA and CRF receptors with i.c.v. injections of the transmitters themselves, effectively induced reinstatement in a manner that would be predicted based on what is known about the neurobiology of footshock-induced reinstatement of cocaine seeking (Shaham et al., 2000a). It is important to note, however, that their effects were not necessarily of the same magnitude, associated with the same variance or time-course, or responsive to the same pharmacological manipulations as footshock stress. For example, i.c.v. injections of NA are associated with a far more modest, but possibly less variable, reinstatement response than are footshock stress or i.c.v. CRF. Likewise, i.c.v. injections of CRF are associated with reinstatement responding that is generally longer lasting than that induced by i.c.v. NA or footshock. Finally, whereas systemic or intra-OFC administration of the D₂-like receptor antagonist, raclopride, failed to block footshock-induced reinstatement of heroin and cocaine, respectively (Shaham and Stewart, 1996; Capriles et al.,

2003), systemic pretreatment with raclopride in the present experiments attenuated CRFinduced reinstatement of cocaine seeking (Chapter 4).

Observations such as these underscore the idea that although specific neurochemical systems can be manipulated in a targeted manner, and induce predictable changes in reinstatement responding, the neurochemical events triggered by different forms of physical or pharmacological stress are not identical. Indeed, the results of Chapter 5 highlight the fact that a single stressor, yohimbine, may act through very different neurochemical systems to mediate reinstatement. As discussed previously in the context of the individual experiments, these differences likely reflect differences (sometimes subtle) in the extent and selectivity of neuronal populations activated by each stressor.

Limitations and Future Directions

In this thesis, I identified functional interactions between CRF and catecholamine systems using systemic (i.p.) and central (i.c.v.) administration of various pharmacological agents. As mentioned, although my findings were largely consistent with what would be predicted about the nature of CRF-catecholamine interactions in stress-induced reinstatement, based on what is known about the neuroanatomical circuits involved in reinstatement, they do not allow conclusions to be made about the loci of interactions. Thus, the next step in this research would be a series of localization studies aimed at manipulating CRF, NA and DA systems within specific regions of interest (e.g., CeA, BNST, VTA or PFC), to demonstrate the nature of their interactions in those regions. This approach would, for example, permit conclusions to be drawn on the functional importance of the CeA and BNST as sites of NA-CRF interaction, as well as the role of D₁- and D₂-like receptor activation in the mPFC on CRF-induced reinstatement of cocaine seeking.

Although such studies are a natural and important extension of the work presented in this dissertation, their feasibility is limited by certain practical considerations. For example, studies of this type would require, in many instances, implantation of four intracranial cannulae in the same animal, to permit bilateral injections of two different drugs (targeting two different receptor populations) in two different brain regions. The feasibility, depending on the regions of interest, of accommodating 4 cannuale in this manner and the ability of animals' tolerating this arrangement alongside the intravenous cannulation, reduce the practicality of this approach.

Given that a functional approach may be constrained by several practical considerations, a correlative approach, detailing the neuroanatomical distribution of specific receptor populations on cells of a particular neurochemical phenotype, may be more viable. Such an approach would, along with the functional studies localizing the effects of individual systems in stress-induced reinstatement, serve to provide a stronger basis for speculating about the nature of CRF-catecholamine interactions in reinstatement. Of course, some of this work has already been done and forms the basis already for the hypotheses on which the present experiments were based. Still outstanding, however, is detailed information about the distribution of different NA receptor subtypes on CRF cell bodies in the CeA and BNST, and detailed information about whether targeted manipulation of those receptors subtypes regulates CRF transmission. To date, neuroanatomical studies have identified the presence of β_1 receptors on CRF-immunoreactive neurons of the CeA (Rudoy et al., 2009), but the expression of α_1 or β_2 -adrenoreceptors on these neurons has yet to be determined; the pattern of NAergic receptor expression on CRF containing cells of the BNST is unknown. From a functional perspective, Leri and colleagues (2002) determined a role for beta-receptors in both the CeA and BNST in footshock induced reinstatement of cocaine seeking; however, a mixed $\beta_{1/2}$ –

adrenoceptor antagonist cocktail of ICI 118, 551 and betaxolol was used, precluding a definite conclusion as to whether β_1 - and/or β_2 -adrenoceptors are specifically involved in reinstatement. Thus, additional information regarding NAergic receptor expression on CRF neurons in the CeA and BNST, as well as the functional involvement of each receptor subtype in the interaction between NA and CRF in these regions will be important to determine in future studies.

Another important question for future research is how the BNST integrates NAergic information from Ltg nuclei and CRF from the CeA or local CRF transmission. In the BNST, antagonism of both CRF and NA receptors blocks the footshock-induced reinstatement of cocaine seeking (Erb and Stewart, 1999; Leri et al., 2002); however, it is currently unknown whether NA and CRF in the BNST act on the same or different populations of neurons (see Figure 3). Given that in this region, NA terminals synapse directly with CRF cells (Hornby and Piekut, 1989; Phelix et al., 1994), and that the BNST provides a source of CRF to the VTA (a projection thought to be involved in the effects of footshock on reinstatement of cocaine seeking [Rodaros et al 2007]), our speculation of a direct interaction between these systems in the stress-induced reinstatement of cocaine seeking would seem reasonable. It is currently unclear, however, which cells in the BNST express CRF receptors, and whether these cells might also express NA receptors. Moreover, the question remains whether NA has its effects on reinstatement in the BNST via its actions on cells containing CRF, or on a different neuronal phenotype entirely. Similar neuroanatomical experiments would be helpful in advancing our understanding of the specific neuronal phenotypes and receptors mediating the interaction between DA and CRF.

The elegant work of Wang and colleagues (2005, 2007) has provided important *functional* information about the nature of CRF-glutamate-DA interactions in the VTA that has

important relevance for understanding mechanisms involved in reinstatement; however the complimentary neuroanatomical data is lacking. For example, it has not been determined whether the CRF receptors involved in activation of VTA DA neurons project to the PFC. One possible approach to investigating this question would be to identify DAergic neurons projecting to the PFC using a retrograde tracing technique, and examine their physiological response to application of CRF.

Another important neuroanatomical issue that needs to be clarified is the distribution of CRF₂ receptors in the VTA. Several *in situ* hybridization studies report relatively low levels of CRF₂ receptor mRNA in the VTA (e.g., Van Pett et al., 2000; Smagin and Dunn, 2000). However, as previously described, the results of several recent microdialysis, reinstatement and electrophysiology studies indicate an important role of activity at CRF₂ receptors in the VTA in mediating the behavioural, neurochemical and physiological response to stress. Thus, studies employing more sensitive mapping techniques, such as immunohistochemistry, would provide important information on the distribution and expression of CRF1 versus CRF2 receptor expression on VTA DA neurons.

Clinical Implications

Despite clinical progress in treating the physical withdrawal syndromes produced by abstinence from opiates, alcohol and nicotine, successful treatments for all drug addictions are either completely lacking or inadequate in controlling drug craving and relapse (Nestler, 2002). An important implication of the present studies, and work conducted by others, is the pivotal role that central extra-hypothalamic CRF systems play in stress-induced reinstatement of drug seeking. Indeed, the present results suggest that CRF systems may be uniquely positioned as a link between the actions of NA in the extended amygdala and downstream effects of the

mesocortical DA system. Thus, it would seem a natural choice to develop pharmacological treatments that target CRF systems. Currently, non-peptide CRF_1 receptor antagonists, such as GSK561679 and GW876008, are being used in clinical trials for major depression, social anxiety disorder and irritable bowl syndrome (Zorrilla and Koob, 2010). To date, an open-label Phase II clinical trial of the non-peptide CRF_1 receptor antagonist R121919 reported reductions in depressive and anxious symptoms in depressed patients (Zorrilla and Koob, 2004). Results from these trials should provide definitive conclusions regarding the therapeutic potential of CRF_1 antagonists for stress-related disorders, and may pave the way for clinical evaluation in addictive disorders.

Conclusion

The results of the experiments presented in this dissertation serve to integrate known information on the independent actions of NA, DA and CRF systems in stress-induced relapse, and elucidate the functional significance of neurochemical interactions between these systems. A basic understanding of the nature of these interactions has important implications for understanding the neurobiological processes that underlie addiction, and the propensity to relapse after extended periods of abstinence. Moreover, the outcome of these studies highlights the systems that should be targeted in the development of therapeutic interventions to treat cocaine addiction.

APPENDIX A

Effects of yohimbine on extracellular DA and its metabolites in the mPFC of animals with

cocaine or saline pre-exposure

Appendix A: Effects of yohimbine on extracellular DA and its metabolites in the mPFC of animals with cocaine or saline pre-exposure

Repeated exposure to psychostimulant drugs, such as cocaine, can produce long-lasting augmentation in subsequent behavioural and neurochemical responses to acute stress. This phenomenon, known as cross-sensitization, is mediated at least in part by neuroadaptations in mesocorticolimbic DA systems. The objective of the experiment presented in this Appendix was to determine whether the pharmacological stressor, yohimbine, elicits the expression of neurochemical sensitization in cocaine pre-exposed rats.

As outlined in Chapter 5, although yohimbine is typically regarded as an α_2 adrenoceptor antagonist, but has also been shown to act on other monoamine systems, including DA. Specifically, yohimbine acts as a antagonist at D₂-like autoreceptors, enhancing DA turnover and inducing DA release in the PFC (Millan et al 2000; Scatton et al 1980). These effects of yohimbine on DA release in mesocortical regions, known to play a role in drug reward, sensitization and reinstatement, are especially noteworthy given the work presented in this dissertation (Chapter 5), and the work of others, showing that at least some of the drug-related behavioural effects of yohimbine are likely mediated by neurotransmitter systems other than NA (Dzung Le et al 2009; Fletcher et al 2008). To this end, I used *in vivo* microdialysis to determine whether rats repeatedly exposed to cocaine (15-30 mg/kg, i.p., for 7 days) would, 10 days later, exhibit sensitized DA release in response to an acute yohimbine challenge (1.25 mg/kg, i.p.), within the mPFC.

As can be seen in the figure below, yohimbine induced a marked enhancement (600% above baseline) in extracellular levels of DA in mPFC, in cocaine relative to saline preexposed rats (Appendix Figure 1, panel A). Similarly, extracellular levels of the metabolites DOPAC and HVA were also enhanced following administration of yohimbine, peaking 197%

and 170% above baseline, respectively; these increases in metabolite levels occurred regardless of whether animals were pre-exposed to cocaine or saline.

Overall, the results confirm and extend previous work demonstrating that chronic treatment with cocaine can result in long-term and regionally specific neuroadaptations of the mesocorticolimbic DA system to acute stress. Moreover, the data is consistent with the idea that effects of yohimbine on drug-related behaviours likely occur via systems other than, and/or in addition to, NA, including DA.

Appendix Figure 1



Mean (\pm SEM) percent baseline levels of extracellular DA (*A*), DOPAC (*B*), and HVA (*C*) before and after administration of 1.25 mg/kg yohimbine (i.p.) in rats previously given i.p. exposures to cocaine or saline.

"Pre YOH" different from "Post YOH" in the cocaine condition, p<.05 (*).

"Pre YOH" different from "Post YOH", p<.01 (##), p<.001 (###).

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