Is it stress? The role of the CRF system and HPA axis, and the identification of brain sites involved in chronic food restriction-induced augmentation of heroin seeking

Firas Sedki

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Chair (Dr. C. Andrew Chapman)

Examiner (Dr. Peter Shizgal)

Examiner (Dr. Wayne Brake)

Supervisor (Dr. Uri Shalev)

Approved by _____

Chair of Department of Graduate Program Director

_____ _____

Date Dean of Faculty

Abstract

Is it stress? The role of the CRF system and the HPA axis, and the identification of brain sites involved in chronic food restriction-induced augmentation of heroin seeking

Firas Sedki
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Drug addiction is a chronic disease characterized by recurring episodes of abstinence and relapse. The mechanisms that underlie this pattern are yet to be elucidated. Recently, we reported that abstinent rats with a history of chronic food restriction show increased heroin seeking compared to sated controls. It is thought that food restriction may cause sensitization of drug seeking due to its stress-like properties, suggesting a critical role for corticotropin-releasing factor (CRF) and corticosterone, hormones involved in the stress response. Blocking corticosterone reduces food restriction-induced sensitization of locomotor activity in response to cocaine, while acute food-deprivation induced reinstatement of extinguished drug seeking is attenuated by CRF antagonism but not removal of corticosterone. The role of CRF and corticosterone in food restriction-induced augmentation of drug seeking remains unknown. Here, male Long-Evans rats were trained to self-administer heroin for 10 days in operant conditioning chambers. Following self-administration rats were subjected to 14 days of unrestricted (sated group) or a mildly restricted (FDR group) access to food, which maintained their body weight at ~75% of the sated rats' body weight. On day 14, rats were administered a selective CRF_1 receptor antagonist (R121919; 0.0, 20.0 mg/kg; IP),

non-selective CRF receptor antagonist (α-helical CRF; 0.0, 10.0, 25.0 μg/μl; ICV) or a glucocorticoid receptor antagonist (RU486; 0.0, 30.0 mg/kg; IP), and underwent a 1 h drug seeking test under extinction conditions. Rats in the FDR group showed a statistically significant increase in heroin seeking compared to the sated group. No statistically significant effects for treatment with R121919, α-helical CRF or RU486 were observed. These findings suggest that stress may not be a critical factor in our paradigm. In an exploratory study to identify brain sites involved in this effect rats were sacrificed post-test and the expression of the immediate early gene, *c-fos*, an indicator of neuronal activity, was measured using immunohistochemistry. Interestingly, a statistically significant decrease in Fos immunoreactivity in the nucleus accumbens shell was observed for the FDR compared to sated rats. Although stress may not be a critical factor in our effect, prolonged exposure to food restriction does cause alterations in reward-related brain sites. The identification of specific neuron types affected in these regions should drive future studies.

Keywords: Heroin, Self-administration, Chronic food restriction, Chronic stress, CRF, Corticosterone, *c-fos*, Nucleus accumbens

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List of Abbreviations

ACTH	Adrenocorticotropic Hormone
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
BLA	Basolateral Amygdala
BNST	Bed Nucleus of Stria Terminalis
BOLD	Blood Oxygen level-Dependent
CeA	Central Nucleus of the Amygdala
CRF	Corticotropin Releasing Factor
CRF_1-R	Corticotropin Releasing $Factor_1$-Receptor
CRF_2-R	Corticotropin Releasing $Factor_2$-Receptor
DA	Dopamine
DAT	Dopamine Transporter
FDR	Food restriction
Fos-IR	Fos Protein Immunoreactivity
GABA	Gamma Aminobutyric Acid
Glu	Glutamate
HPA axis	Hypothalamic-Pituitary-Adrenal axis
il-PFC	Infralimbic Cortex
KO	Knockout
mPFC	Medial Prefrontal Cortex
NAc	Nucleus Accumbens
NAcC	Nucleus Accumbens Core
NAcS	Nucleus Accumbens Shell
NMDA	N-Methyl-D-aspartate
OFC	Orbitofrontal Cortex
PFC	Prefrontal Cortex
pl-PFC	Prelimbic Cortex
PVN	Paraventricular Nucleus
SN	Substnatia Nigra
VTA	Ventral Tegmental Area

General Introduction

Drug addiction is a chronic disease characterized by recurring episodes of abstinence and relapse. Despite negative health, social, and psychological consequences of drug abuse, nearly 200,000 Canadians are dependent on illicit drugs, accruing costs of over eight billion dollars per year in medical care, crime-related damages and expenses, and the toll of addressing other social problems (Health Reports, 2004). These are significant but the perception of the "recreational", non-therapeutic nature of drug-uses can have a negative impact on the progress of treatment efforts (McLellan, 2000). As a result, treatment for drug addiction remains widely ineffective. As many as 80% of abstinent drugs users return to active substance use within one year following treatment (Hser, Grella, Shen, & Anglin, 2000), providing further evidence that the treatment of drug addiction remains inadequate.

Treatment of relapse typically involves acute, rescue pharmacological intervention and thus reflects a poor model of chronic disease management (White, Boyle, & Loveland, 2002). While relapse may occur shortly after drug cessation, it is not the presence of withdrawal symptoms that precipitates drug relapse. Instead, it is the subjective craving and the urge to seek out the drug (Robinson & Berridge, 1993; Wise & Bozarth, 1987). In fact, abstinent drug users often report an incubation period, whereby drug craving increases over time (Gawin & Kleber, 1986), contrary to the expectation that it will diminish as time passes. Thus, relapse may occur following many months or years of abstinence (Hser, Hoffman, Grella, & Anglin, 2001).

It is believed that a neurobiological approach to drug addiction is critical to develop efficient research-directed treatments for addiction, particularly since drugs of

abuse have been shown to alter neural structures, functions, and brain chemistry. These alterations may affect the motivational and cognitive processes that are fundamental to relapse (Koob & Bloom, 1988). The persistent changes in brain chemistry may also increase vulnerability to triggers that induce relapse, such as re-exposure to drug-associated cues and environments (Childress et al., 1993), to the drug itself (De Wit, 1996), and to stressful situations (Sinha, 2001).

Drug users consistently report stress as a factor in subjective craving as well as in the initiation, maintenance and relapse of drug use (Brewer, Catalano, Haggerty, Gainey, & Fleming, 1998; Matheny & Weatherman, 1998; Sinha, 2001; 2008; Sinha & O'Malley, 1999). The definition of "stress" has proven to be difficult, but for the purpose of our discussion, stress can be defined as the experience of negative emotions combined with predictable changes in physiology, biochemistry, behavior, and cognition (Baum, 1990). For stress to remain manageable, physiological systems operate to suppress unnecessary functions and activate those required for immediate survival (Kemeny, 2003). While adaptive upon exposure to short-term stressors, chronic over-activation can disrupt these systems and negatively alter reactions to stress (McEwen, 1998). Thus, although performance in demanding tasks is facilitated by mild, short-term stress, negative, uncontrollable, unpredictable and chronic stress renders such goal-oriented tasks unmanageable or overwhelming (Lazarus, 1999; Levine, 2005; Lovallo, 2005; McEwen, 2002; Meaney, Brake, & Gratton, 2002; Selye, 1984). In addition, threat is experienced when the demands in a given situation are perceived to outweigh the resources available to that individual (Blascovich, 1996). It is this negative psychological or physiological stress that may trigger a return to drug use (Back et al., 2010; Sinha, 2001; 2008).

A role for stress as a potential trigger for relapse to drugs has been identified both in retrospective studies involving interviews and questionnaires given to addicts, and in controlled laboratory studies. For example, studies by Kosten and colleagues (1983; 1986) suggest that stress is associated with relapse, as opioid users reported experiencing a greater number of stressful life events when compared to healthy controls. A different approach involves the exposure of abstinent addicts to structured, individually adjusted scripts describing highly relevant stressful experiences in laboratory settings, resulting in increased subjective drug-craving (Sinha, Garcia, Paliwal, Kreek, & Rounsaville, 2006).

While a role for stress in drug abuse and relapse is clearly indicated, the mechanisms that underlie this effect have not been completely elucidated. Both clinical and pre-clinical studies have demonstrated that stress can activate reward-related brain regions, while drugs of abuse can activate stress-related brain regions (Bossert, Ghitza, Lu, Epstein, & Shaham, 2005; Koob, 2008; Sinha, 2008). These findings suggest a neurobiological link between stress and abused drugs (Sinha, 2001; 2007). One explanation, therefore, is that stress activates brain reward circuitry, resulting in an increased sensitivity to the reinforcing properties of abused drugs, leading to a greater motivation for drug seeking (Piazza & Le Moal, 1998; Sinha, 2009). Drug use can also cause the structural and chemical modification of neural pathways, producing an increasingly negative response to stress, and therefore further motivating drug seeking behavior (Sinha, 2001; 2005).

The aforementioned studies suggest a critical role for stress in driving drug use and consequently drug relapse in human subjects. However, the study of relapse in humans is difficult and fraught with ethical obstacles. To elucidate the underlying

neurobiological mechanisms involved in drug craving, motivational processes and relapse, the use of animal models is crucial.

One of the most frequently used models for drug relapse in recent years is the self-administration-based reinstatement procedure. Briefly, animals are trained to perform an operant response to self administer a drug. Successful drug administration is often paired with the presentation of discrete cues (e.g., light or tone). The drug seeking behavior is then extinguished by the removal of the drug while keeping all other conditions similar to training. The animals can then be exposed to any of the conditions known to trigger craving and relapse in humans (de Wit & Stewart, 1981; Shaham, Shalev, Lu, & de Wit, 2003). Accordingly, reintroduction to a previously drug-paired environmental context, exposure to discrete drug-associated cues or to a small dose of a previously administered drug (e.g. heroin, cocaine) and exposure to a stressor such as electrical footshock stress, have all been shown to successfully reinstate extinguished drug-seeking (Shalev, Grimm, & Shaham, 2002; Stewart, 2000).

Using the reinstatement procedure, significant advances in uncovering the neuronal circuitry underlying stress-induced reinstatement has been made. Reports from Shaham and colleagues (1997) strongly implicate stress-induced activation of the corticotropin-releasing factor (CRF) system in the reinstatement of drug seeking. CRF, which is synthesized and released by the hypothalamus and extra-hypothalamic brain regions, is a main component in the behavioral and physiological response to stress (DeSouza, 1995; Koob, Heinrichs, Menzaghi, Pich, & Britton, 1994; Turnbull & Rivier, 1997). It was demonstrated that CRF receptor antagonism attenuates electrical footshock-induced reinstatement of heroin seeking and cocaine seeking (Shaham et al., 1997). In

contrast, the removal or blockade of corticosterone, the product of stress-induced activation of the hypothalamus-pituitary-adrenal (HPA) axis, does not attenuate footshock-induced reinstatement, thus providing evidence that CRF may be acting on extra-hypothalamic targets rather than on the activation of the HPA axis (Shaham et al., 1997).

A second major component in the central stress response is the activation of noradrenergic (NA) projections from the brain stem. The dorsal NAergic bundle projects from the locus coeruleus (LC), to cortical brain regions (Moore & Bloom, 1979), and is involved in the stress response (Tanaka et al., 1990). Shaham and colleagues (2000b), however, reported no effect of intra-LC infusions of the α-2 adrenoreceptor agonist clonidine on footshock-induced reinstatement of heroin seeking. Alternatively, the ventral NAergic bundle (VNAB) involves projections from the lateral tegmental nuclei, to brain regions such as the central extended amygdala (CeA) and nucleus accumbens (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Fritschy & Grzanna, 1991; Moore & Bloom, 1979). This pathway may play a more prominent role in stress-induced reinstatement of drug seeking , as 6-Hydroxydopamine lesions of the lateral tegmental area attenuated footshock-induced reinstatement (Shaham et al., 2000b).

The CRF and NAergic brain systems may interact to modulate footshock-induced reinstatement. Erb et al. (Erb & Stewart, 1999; Erb, Salmaso, Rodaros, & Stewart, 2001), have described a pathway involving the activation of CRF neurons in the CeA by stress-induced NE release, which in turn act on CRF receptors in the BNST and consequently drive footshock-induced reinstatement of drug-seeking (Shalev et al., 2002).

As mentioned above, stress and reward pathways in the brain seem to interact to produce the affective and behavioral aspects of the stress response. The mesolimbic dopamine (DA) pathway which consists of the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and to the prefrontal cortex (PFC), is implicated in reward processing (Kelley et al., 2002). It is thought that rewarding properties of all drugs of abuse relate to their ability to generate, either directly or indirectly, a release of DA in this pathways (Wise, 1998). In addition, an overflow of DA in the NAc is observed following footshock-induced reinstatement of heroin seeking (Shaham & Stewart, 1995). In agreement with the suggested role of the DAergic system in stress-induced reinstatement, systemic administration of a mixed dopamine antagonist, flupenthixol attenuates, footshock-induced reinstatement of heroin seeking (Shaham & Stewart, 1996). Surprisingly, administration of specific DA D1 or D2-like receptor antagonists did not significantly affect footshock stress-induced reinstatement (Shaham & Stewart, 1996). However, intracranial infusions of SCH-23390, a D1-like receptor antagonist, but not a D2-like receptor antagonist, into the prelimbic or orbitofrontal cortex blocked footshock-induced reinstatement of cocaine seeking (Capriles, Rodaros, Sorge, & Stewart, 2003), suggesting a critical role for DA transmission in these areas in stress-induced reinstatement.

It is possible that the CRF and DA brain systems interact to drive stress-induced drug seeking behavior. Intra-VTA infusions of a selective CRF1 receptor antagonist, for example, block footshock-induced reinstatement of cocaine seeking (Blacktop et al., 2011). In addition, it has been shown that CRF is released in the VTA following exposure to footshock stress (Wang et al., 2005). This release of CRF may in turn modulate

glutamate (an excitatory neurotransmitter) release in the VTA, as well as activation of the mesolimbic dopamine system. This effect was found to be selective to rats with a history of cocaine taking versus naive controls (Wang et al., 2005). It was therefore suggested that footshock-induced reinstatement is mediated by a coordinated activation of the CRF and DAergic circuitry.

The studies described above have contributed a remarkable amount of knowledge on the link between stress and relapse, and to the elucidation of the underlying brain mechanisms. We, however, believe footshock to be too extreme of an environmental stressor, which is hardly relevant to the human condition. We have therefore focused on the effects of a more biologically relevant environmental manipulation, exposure to caloric restriction. Dietary restriction causes physiological and negative affective states that result in increased cortisol levels (Tomiyama et al., 2010) and disrupts neuronal pathways that subsequently might augment drug use in human subjects (Franklin, Burtrum, Brozek, & Keys, 1948; Hall, Tunstall, Vila, & Duffy, 1992; Hanna & Hornick, 1977; Krahn, Kurth, Demitrack, & Drewnowski, 1992). In times of war, dietary restriction was linked to increases in nicotine and caffeine consumption (Franklin et al., 1948). Also, malnourished Peruvian Indians were shown to chew more coca leaves (Hanna & Hornick, 1977). An increased risk for relapse among calorically restricted abstinent smokers (Hall et al., 1992), as well as a positive correlation between the severity of diet and the risk of drug taking in young women (Krahn et al., 1992), suggest a strong link between dietary restriction and drug intake.

Given the demonstrated impact on human drug users (Franklin et al., 1948; Krahn et al., 1992), the high comorbidity with eating related disorders (Holderness & Gunn,

1994), and their ability to cause disruptions of the stress system, dietary manipulations such as acute food deprivation (FD) and food restriction (FDR) seem to be highly relevant to the study of relapse. In laboratory animals, the effects of dietary manipulations on drug-associated behaviors has been unequivocally demonstrated. The initiation and maintenance of drug relapse is reliably enhanced following periods of food deficiency (acute FD and food restriction) in the rat (Lu, Shepard, Scott Hall, & Shaham, 2003; Piazza & Le Moal, 1998). In addition, modifications of reward and stress related mechanisms implicated in the resumption of drug seeking, the CRF and DA systems, are observed following dietary restriction (Carr, 2002; Shalev, Robarts, Shaham, & Morales, 2003b). Importantly, acute food deprivation (24-48 h), can induce reinstatement to drug seeking in rats with a history of heroin or cocaine self-administration (Shaham et al., 2003; Shalev, Highfield, Yap, & Shaham, 2000).

As with other stressors, food deficiency can increase corticosterone release in rodents (Dallman et al., 1999; Marinelli, Le Moal, & Piazza, 1996). In addition, a pharmacological blockade or removal of corticosterone can attenuate food restriction-induced augmentation of the locomotor enhancing effects of psychostimulant drugs (Deroche et al., 1995; Marinelli et al., 1996). However, in line with previous findings by Shaham and colleagues (1997), CRF receptor antagonism, but not the removal of corticosterone, attenuated acute FD-induced reinstatement of drug seeking (Shalev, Finnie, Quinn, Tobin, & Wahi, 2006). Finally, similar to footshock-induced reinstatement, acute FD-induced reinstatement of drug seeking involves the dopaminergic system. However, unlike the former, acute FD-induced reinstatement of heroin seeking was attenuated by the systemic administration of the selective DA D1-like, SCH 23390,

but not the D2-like, receptor antagonist (Tobin, Newman, Quinn, & Shalev, 2009), suggesting that different neuronal mechanisms might underlie reinstatement induced by these two stressors.

Although acute FD and food restriction can decrease body weight and augment drug seeking in rodents, such similarities are accompanied by differential metabolic and behavioral effects. For example, gene expression of neuropeptide Y, a peptide known to regulate energy balance in response to food intake, is increased in the dorsomedial hypothalamus following chronic food restriction but not acute food deprivation (Bi, Robinson, & Moran, 2003). Furthermore, Fulton and colleagues (2000) demonstrated a decreased threshold for electrical brain stimulation reward in chronically food restricted, but not acutely food deprived, rats; this effect was attenuated by leptin, a hormone involved in energy balance and metabolism. These studies suggest that differential underlying mechanisms may exist between food restriction and food deprivation. Additionally, recent studies report an increased risk for relapse among abstinent smokers following prolonged food restriction, and not acute food deprivation, suggesting the need for a more ecologically valid model of drug relapse (Cheskin, Hess, Henningfield, & Gorelick, 2005).

The intuitive appeal of the reinstatement model, stemming from the fact that the main factors known to trigger relapse in humans also induce reinstatement of drug seeking in laboratory animals, has made it the most used animal model of drug relapse (Epstein, Preston, Stewart, & Shaham, 2006). Nevertheless, we contend that human addicts rarely undergo explicit periods of extinction. Alternatively, addicts experience short or prolonged periods of abstinence and withdrawal before the resumption of drug

seeking. In addition, recent studies have outlined important differences in the neural circuitry involved in abstinence versus extinction learning (Fuchs, 2006; Fuchs, Lasseter, Ramirez, & Xie, 2008a). Inactivation of the caudate putamen (Cp), for example, attenuates cocaine-seeking following a period of abstinence. However, inactivation of other structures that are implicated in the reinstatement of extinguished drug seeking was without effect (Fuchs, 2006).

Consequently, we suggest a more clinically relevant model, where rats with a history of heroin self-administration are tested following prolonged food restriction and abstinence. Our model consists of three phases: animals are first trained to self-administer a drug in the presence of a cue/tone complex (training phase), then moved to a different context and undergo a prolonged period of FDR or remain sated (abstinence phase) and finally returned to the self-administration environment for a reward-seeking test in presence of drug-paired cues under extinction conditions (test phase). Using this revised model, our laboratory has recently reported a dramatic (> 250%) enhancement of heroin-seeking in food restricted rats, compared to sated controls (D'Cunha, Sedki, Macri, Casola & Shalev, 2012). Preliminary evidence suggests an association between changes in DA release and the augmentation of heroin seeking following chronic food restriction. The role of the DA system in this effect is the subject of an ongoing study in our laboratory.

Elucidating the mechanisms involved in the food restriction-induced augmentation of drug seeking would have a significant contribution to the understanding of the way environmental challenges might drive relapse to drugs. Therefore, in the experiments described in Chapter 1 of this thesis we investigated the role of stress

systems, and more specifically, the involvement of CRF and corticosterone, in the augmentation of drug seeking following prolonged food restriction and abstinence. In Chapter 2, we report findings with Fos protein immunoreactivity to identify neuronal activation in reward and stress related brain regions, following exposure to prolonged food restriction and abstinence period. Finally, despite extensive efforts to identify the underlying mechanisms involved in drug reward and food restriction, the literature tends to target psychostimulant drugs and extinction paradigms, with little emphasis on the effects of opiates. Recent reports have outlined the importance of differentiating the interpretations made in research using opiates versus psychostimulant drugs (Badiani, Belin, Epstein, Calu, & Shaham, 2011). Therefore the studies described here involve rats that have been trained to self-administer heroin, an opiate drug.

General Methods

Subjects

Male, Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 300-350g) were used. Before surgery, animals were pair-housed for one week in the animal care facility (ACF) under reverse light/dark conditions (lights OFF at 09h30). Following intravenous (IV) catheterization, and two days of recovery, rats were single-housed in plastic shoebox cages before being transferred to operant conditioning chambers for drug self-administration. Following self-administration training, rats were returned to the ACF and single-housed in shoebox cages for the abstinence phase. Except for the abstinence phase, all rats were given unrestricted access to food and water. Rats were treated according to the Canadian Council on Animal Care guidelines, and approval was granted by the Concordia University Animal Research Ethics Committee.

Surgical procedures

Rats were implanted with IV silastic catheters (Dow Corning, Midland, MI, USA) under xylazine/ketamine (10+100 mg/kg; i.p.). Three centimeters of silastic catheter was inserted through a small incision on the right jugular vein, and secured using silk sutures. The remainder of the catheter was passed subcutaneously to the skull, attached to a modified 22-gauge cannula (Plastics One Industries, Roanoke, VA) and anchored to the skull using dental cement and 5 jeweler's screws. Post-surgery, animals were administered buprenorphine (600 ug/rat; Schering-Plough Ltd., Welwyn Garden City Hertfordshire, UK) and penicillin (450,000 IU/rat) to reduce pain and prevent infection. Catheters were flushed daily with heparin/gentamicin (7.5 IU + 12.0 mg/rat) to prevent blockage and infection.

Apparatus

Operant conditioning chambers. Rats were housed individually in operant-conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm x 29.0 cm x 25.5 cm) enclosed in sound attenuating wooden compartments equipped with a fan. Each chamber consisted of a stainless steel metal grid floor, a front and back Plexiglas wall and two metal panel sidewalls. Two retractable levers (Coulbourn Instruments) were mounted 9 cm above the floor of the right sidewall. Responding on the drug-paired (active) lever activated the infusion pump while responding on the non-drug-paired (inactive) lever had no programmable consequences. A cue-light (Coulbourn Instruments) and tone module (Sonalert, 2.9 KHz, Coulbourn Instruments) were located above the active lever, and a red house-light was positioned at the top-center of the left sidewall. The drug pump was connected to the catheter via a liquid swivel (Instech Swivel Assembly, Boulder, CO,

USA), and Tygon tubing (Norton Performance Plastics, Akron. OH, USA; OD 0.06, ID 0.02) protected with a metal spring.

Drug

Heroin hydrochloride (HCl) (a contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in sterile saline (5.00 mg/ml) and then further diluted with saline, for each rat (0.10 mg/kg/infusion).

Procedure

Self-administration. Following a 24-h habituation period in the operant chamber, rats were trained to self-administer heroin for 10 days with three 3-h sessions separated by 3-h intervals. Each daily session began shortly after the onset of the dark phase with an extension of the active and inactive levers, illumination of a house-light and activation of the cue-light/tone complex for 30 s. Responses on the active lever, which was armed with a fixed ratio-1 schedule (FR-1), resulted in activation of the drug pump (5 s, 0.13 ml/infusion) and the initiation of a 20 s timeout during which the house-light was turned off and the cue light/tone complex above the active lever was activated. During the timeout period, active lever responses were recorded but not reinforced. Following each 3-h session, the active lever was retracted whereas the inactive lever was not retracted until 1 h before the first session of the following day. Inactive lever responses were recorded but had no programmable consequences.

Abstinence Phase. Following self-administration training, rats were individually housed in the ACF, and given unrestricted access to food and water for one drug-washout day. Rats were then divided into two groups: food restricted (FDR) or Sated, that were

matched according to body weight, and number of infusions and active lever responses across the last 5 days of training. Following the washout day, FDR rats had their food removed and were fed approximately 15 g of rat chow at 13h30. The amount of food was adjusted through 14 days of food restriction to maintain the food restricted rats' body weight (BW) to approximately 75-80% of the Sated rats and 90% of their baseline BW.

Test Phase. On the morning of abstinence day 14, rats were returned to the operant conditioning chambers and attached to the metal spring. The test phase consisted of a 1-h session during which active lever responses had the same consequences as in training excluding the availability of the drug (see Figure A1 for a timeline of events).

Chapter 1

The role of stress systems in chronic food restriction-induced augmentation of heroin seeking in the rat.

Sedki, F., Abbas, Z., Martin, J., D'Cunha, T. & Shalev, U.

Introduction

Stress is often cited as a precipitating factor in drug relapse (Brewer et al., 1998; Matheny & Weatherman, 1998; Sinha, 2001; 2008; Sinha & O'Malley, 1999). However, the precise mechanisms underlying this effect are not clear. The corticotropin releasing factor (CRF), through its actions on hypothalamic and extra-hypothalamic sites, plays a critical role in the behavioral and physiological response to stress. Based on the elevation of CRF concentrations in the cerebrospinal fluid of clinically depressed patients, it is suggested that CRF plays an active role in the modulation of stress and reward-related processes (Kehne, 2007). Studies by Heim and colleagues (2008) provide evidence that dysregulation of the CRF system in early childhood can cause long-lasting sensitization of CRF-mediated stress responses in adults. In addition, manipulations of the CRF system exhibit therapeutic value, as treatment with R121919, a selective CRF_1 receptor (CRF_1-R) antagonist, can attenuate depressive symptoms (Künzel et al., 2003; O'Brien, Skelton, Owens, & Nemeroff, 2001; Zobel et al., 2000). Interestingly, this effect seems to be mediated by the actions of treatment on extra-hypothalamic brain regions, as activity of the hypothalamic-pituitary-adrenal (HPA) axis stress response remained unaltered during testing (Künzel et al., 2003; Zobel et al., 2000).

CRF is a 41-amino acid neuropeptide, with two known receptor subtypes: CRF_1-R and CRF_2 -R. Within the HPA axis, the paraventricular nucleus of the hypothalamus releases CRF following exposure to stress. At the pituitary gland, CRF acts on the CRF_1-Rs to trigger the synthesis of the adrenocorticotropic hormone (ACTH). ACTH release results in the output of cortisol and corticosterone from the adrenal glands. This activates

the body's "fight or flight" response, and drives the body into a state of hyper-alertness (Miller & O'Callaghan, 2002).

As mentioned before, CRF actions at extra-hypothalamic brain regions might be particularly important for its effects on affective and motivational processes. CRF positive neurons are found at high densities in the central extended amygdala (CeA), bed nucleus of stria terminalis (BNST), brainstem, striatum, ventral tegmental area (VTA), and cerebral cortex (Charlton, Ferrier, & Perry, 1987; Cummings, Elde, Ells, & Lindall, 1983; Swanson, Sawchenko, Rivier, & Vale, 1983; Udelsman et al., 1986; Vale, Spiess, Rivier, & Rivier, 1981). The distribution of CRF_1-R mRNA is reported across the cerebral cortex, basolateral amygdala, medial amygdala, medial septum and BNST, with limited expression in the nucleus accumbens and VTA (Potter et al., 1994; Van Pett et al., 2000). In contrast, CRF_2-Rs are predominantly found in subcortical regions; the choroid plexus, lateral septum and medial BNST (Bittencourt & Sawchenko, 2000; Chalmers, Lovenberg, & De Souza, 1995). Circulating CRF posses a greater affinity for CRF_1 and not CRF_2-Rs. Both, however, are critical to the stress response (Dautzenberg & Hauger, 2002; Hauger, Risbrough, Brauns, & Dautzenberg, 2006; Hauger, Risbrough, Oakley, Olivares Reyes, & Dautzenberg, 2009; Perrin & Vale, 1999; Reul & Holsboer, 2002), as CRF_2-R activation counteracts increased anxiety-like behaviors observed following CRF_1-R activation (Risbrough, Hauger, Roberts, Vale, & Geyer, 2004).

CRF_1-R knockout (KO) mice exhibit diminished anxiety-like behaviors. In contrast, CRF_2-R KO mice displayed increased levels of anxiety-like behaviors (Bale et al., 2000; Kishimoto et al., 2000); however contrasting findings were also reported, with CRF_2-R KO mice demonstrating an attenuated stress response (Coste et al., 2000).

Despite a lack of acute changes in anxiety-like behaviors, CRF_2-R KO mice exhibited impaired responding under prolonged exposure to stress, which suggests limited capabilities in long-term adaptations to stress (Coste et al., 2000; Logrip, Koob, & Zorrilla, 2011). These data outline a clear role for CRF_1-Rs in the augmentation of stress responsivity (Koob & Heinrichs, 1999). CRF_2-Rs may play a critical role in adaptation to stress, however their role remains unclear and dependent upon differences in research methodology (Fekete & Zorrilla, 2007; Ho et al., 2001; Takahashi, Ho, Livanov, Graciani, & Arneric, 2001; Zhao et al., 2007). The following study will therefore focus on the involvement of the CRF system, and specifically the CRF_1-Rs, in drug seeking.

The appeal of studying the role of the CRF system in drug addiction is derived from the fact that abused drugs have the potential to activate a stress response in the body (Sarnyai, 1998; Sarnyai, Shaham, & Heinrichs, 2001), and as discussed in the general introduction, exposure to stress is associated with increased drug-taking and drug seeking in human subjects (Brown, Vik, & Patterson, 1995; Shiffman & Wills, 1985) and laboratory animals (Piazza & Le Moal, 1996; Shaham, Erb, & Stewart, 2000a). For example, rodents given extended, but not short-access to cocaine, nicotine, ethanol, or heroin self-administration display increased drug taking over time, which can be attenuated with treatment of a CRF_1-R antagonist (Funk, Zorrilla, Lee, Rice, & Koob, 2007; George et al., 2007; Greenwell, Funk, Cottone, Richardson, Chen, Rice, Zorrilla, & Koob, 2009a; Specio et al., 2007). With respect to drug relapse, administration of a non-selective CRF-R or selective CRF_1-R antagonist can block stress-induced reinstatement in rats with a history of cocaine, heroin, alcohol or nicotine self-administration

(Bruijnzeel, Prado, & Isaac, 2009; Erb, Shaham, & Stewart, 1998; Gehlert et al., 2007; Shaham et al., 1997; Shalev et al., 2006).

A common feature shared by all drugs of abuse are somatic withdrawal symptoms, an over activation of the physiological stress response (HPA-axis) system and increased CRF release from the amygdala. Repeated drug use and prolonged periods of abstinence, however, results in a blunted HPA-axis response and an increase in anxiety-like behaviors. In part, this response is caused by the sensitization of the extra-hypothalamic CRF stress system (Koob, 2008; Koob & Kreek, 2007). These adaptations in the extra-hypothalamic CRF system are accompanied by increased drug craving and drug seeking behaviors (Bossert et al., 2005; Bruijnzeel & Gold, 2005; Koob & Zorrilla, 2010; Logrip et al., 2011). Zorilla and colleagues (2001) report changes in CRF tissue-content in the amygdala, following protracted abstinence from ethanol or cocaine. In addition, exposure to restraint stress in rats which have previously self-administered ethanol increases sensitivity and anxiety-like behaviors following a six week abstinence period, an effect that was attenuated upon treatment with the non-selective CRF-R antagonist, D-Phe CRF_{12-41} (Valdez, Roberts, Chan, Davis, Brenna, Zorilla, & Koob, 2002). This provides evidence that abstinence is a dynamic condition, where long-lasting changes in neural circuitry continue to occur over time. Moreover, various brain adaptations that occur over abstinence have been associated with an increase motivation for drug seeking, termed incubation of drug craving, in both humans and animals (Bedi et al., 2011; Conrad et al., 2008; Grimm, Hope, Wise, & Shaham, 2001; Lu, Grimm, Hope, & Shaham, 2004). Incubation of drug seeking has also been demonstrated with stress-induced reinstatement of drug seeking. Thus, footshock-induced reinstatement of heroin

seeking was higher following 12 or 25 days, compared to one day of abstinence (Shalev, Morales, Hope, Yap, & Shaham, 2001a).

Recently, we have demonstrated an augmentation of heroin seeking in abstinent, chronically food-restricted rats (D'Cunha et al., 2012). The findings presented above, and in the general introduction, suggest that over the period of abstinence, exposure to food restriction modulates brain adaptations, possibly in stress-related pathways, resulting in the augmentation of drug seeking. In the study presented here we investigated the role of CRF transmission and HPA axis activation in the augmentation of heroin seeking following exposure to 14 days of food restriction in abstinent rats. In the first experiment, we studied the effects of acute treatment with the selective CRF_1-R antagonist R121919 or the non-selective CRF-R antagonist, α-helical-CRF before a drug seeking test on food restriction-induced augmentation of heroin seeking. The second experiment describes the effects of treatment with RU486, a glucocorticoid receptor antagonist, on heroin seeking in food restricted, abstinent rats. It was expected that the antagonism of CRF-Rs, more specifically of CRF_1-Rs would attenuate the augmentation in heroin seeking that is seen in rats following chronic food restriction when compared to sated controls. We did not expect that the blockade of glucocorticoid receptors would attenuate heroin seeking in the food restricted or sated groups as previous research indicated that the corticosterone system is not involved in a food deprivation induced reinstatement of heroin seeking (Shalev et al., 2006).

Experiment 1: The role of CRF in chronic food restriction-induced augmentation of heroin seeking in the rat.

Methods

Subjects

Seventy-seven male, Long-Evans rats (Charles River, St. Constant, Quebec, Canada; Harlan Laboratories, IN, USA, 300-350g) were used. Rats were housed and treated as described in the general methods.

Surgical procedures

As described in the general methods, rats were implanted with IV silastic catheters to allow for drug self-administration. Some rats (experiment 1B) were also implanted with a 22-gauge guide cannula (Plastics One, Roanoke, VA) aimed 2 mm above the right or left lateral ventricle (AP, ± 0.5; ML, +1.4; DV, −3.0; relative to bregma) to allow for intracerebroventricular (ICV) infusions. Both cannulae were subsequently anchored to the skull using dental cement and 5 jeweler's screws. Catheters were flushed daily with heparin/gentamicin to prevent blockage and infection

Apparatus

Operant conditioning chambers. The operant conditioning chambers used were identical to those described in the general methods.

Drug

Heroin HCl was prepared as described in the general methods. Experiment 1A: R121919, the selective CRF_1 receptor antagonist, was kindly supplied by Dr. Kenner Rice (National Institute on Drug Abuse, NIH, Baltimore, MD, USA). R121919 was

dissolved in a 20% β-Cyclodextrin (Sigma-Aldrich) sterile saline solution to a concentration of 10.0 mg/kg and adjusted to a pH of 4.5. R121919 was injected at a final dose of 20.0 mg/kg. A solution of 20% β-Cyclodextrin mixed in sterile saline solution was used as a vehicle (0.0 mg/kg). The doses for R121919 are based on previous reports that observed red (Greenwell, Funk, Cottone, Richardson, Chen, Rice, Zorrilla, & Koob, 2009b; Gutman, Owens, Thrivikraman, & Nemeroff, 2010)

Experiment 1B: The non-selective CRF antagonist, α-Helical CRF (Sigma-Aldrich), was dissolved in sterile water to a concentration of 5.0 µg/µl or 12.5 µg/µl. α-Helical CRF was infused over 2 minutes at a rate of 1.0 µg/min for a final dose of 10.0 or 25.0 µg/rat, ICV. Sterile water was used as a vehicle. The injector (28-gauge, Plastics One) extended 2 mm below the implanted guide cannula and was kept in place for 60 s after the injection to allow for proper diffusion of the drug. The doses for α-Helical CRF are based on previous reports (Baldwin, Rassnick, Rivier, Koob, & Britton, 1991; Krahn, Gosnell, Grace, & Levine, 1986; Shalev et al., 2006).

Procedure

Self-administration and abstinence phase procedures were similar to those described in the general methods, with the exception that for rats in experiment 1B (α-Helical CRF infusions), guide cannula placements were verified using an angiotensin II-induced (100.0 nmol, ICV) short latency (<60 s) drinking response.

Test Phase. On day 14 of abstinence, in experiment 1A, each rat was given a subcutaneous injection of R121919 (0.0, 20.0 mg/kg) 30 min before the test session. For

experiment 1B, an ICV infusion of α-Helical CRF (0.0, 10.0 or 25.0 µg/rat) was administered 10 min before the test session.

Experiment 1B, Anxiety Test. Since treatment with CRF receptor antagonists did not have any effect on heroin seeking following abstinence, we examined the efficacy of the treatment using a different paradigm. Previously, α-Helical CRF has reliably shown anxiolytic properties (Koob & Heinrichs, 1999). An anxiety test was conducted to ensure that α-Helical CRF was effective in decreasing stress and anxiety levels in test rats. Eight rats that were fitted with ICV guide cannula and participated in the drug-seeking study, were restricted for an additional 8 days following the test phase. On day 8, at 13h30 rats were brought into a novel, brightly lit, environment and placed in a white circular arena (diameter 137 cm; height 46 cm) with one food pellet in the center. Rats where placed at either the north, south, west or east positions of the arena and allowed to explore the environment for 10 min. The rats' behavior was recorded by a video camera. Two variables were then scored from the video recordings. The first, latency to consumption, was defined as the time for the rat to first consume a portion of the food pellet. The second, was number of approaches defined as the number of times a rat approached the food pellet until first consumption.

Statistical Analysis

All analysis were conducted using SPSS software (IBM, SPSS Statistics, version 20). Training data for all rats were analyzed using a within subjects ANOVA, with *training day* (1-10) as the independent variable and active lever responses, inactive lever responses or number of infusions as the dependent variable.

Number of responses on the active and inactive levers during the test session were analyzed using separate univariate ANOVAs, with *antagonist dose* (experiment 1A: 0.0, 20.0 mg/kg; experiment 1B: 0.0, 10.0, 25.0 µg/kg) and *feeding condition* (FDR, Sated) as the independent variables.

Two independent samples t-tests were carried out to test the efficacy of α-Helical CRF in the anxiety test. The independent variable was *antagonist dose* (0.0, 25.0 µg/kg) of α-Helical CRF. Latency to consumption or number of approaches were the dependent variable. The critical cut off point for statistically significant results was $p \leq .05$, unless otherwise stated.

Results

Experiment 1A - The Effects of Treatment with the Selective CRF_1 Receptor Antagonist, R121919, on Chronic Food Restriction-induced Augmentation of Heroin Seeking in the Rat

Final analysis included 25 rats. A total of 5 rats were removed due to catheter leakage, failure to train, or detached head-cap. The remaining rats acquired reliable heroin self-administration behavior.

Training. Mauchly's test of sphericity assumptions were violated for all training data. All values recorded were corrected using the Greenhouse-Geisser correction. A statistically significant increase in heroin infusions over time was observed, $F(9,216) = 10.76$, $p < .001$. Active lever responding increased across training sessions, while there was no statistically significant change in inactive lever responding over time, $F(9,216) = 10.73$, $p < .001$ (see Figure 1). Following the heroin self-administration phase, rats were

separated into two groups FDR (n=12) or Sated (n=13) that were matched according to the mean number of infusions, active lever responding and body weights over the last five days of training. Data on the last day of training is as follows; mean number of infusions (M = 42.08, SEM = 4.25), active and (M = 155.72, SEM = 18.97) inactive lever (M = 13.28, SEM = 3.36) responses.

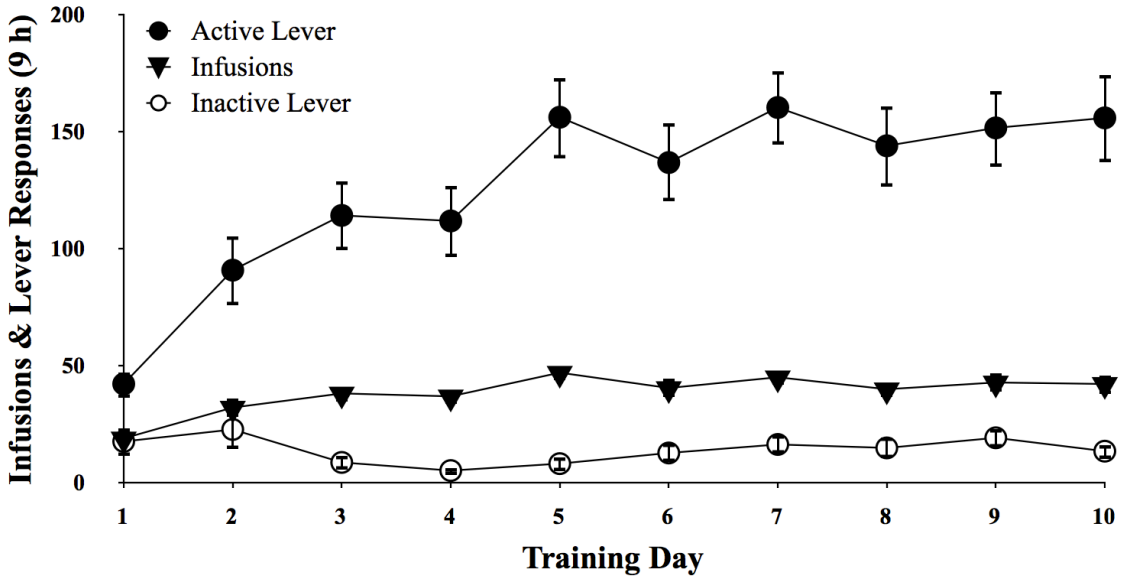

Figure 1. Mean (± SEM) number of infusions, active, and inactive lever responses made during heroin self-administration training by rats in experiment 1A (n = 25). Heroin (0.1 mg/kg/infusion) was self-administered in three 3-h sessions, over 10 days, under a FR1 + 20 s time out schedule of reinforcement. Infusions and active lever responding increased at a statistically significant level ($p < .05$) over training days, whereas inactive lever responding did not.

Test. The number of rats in each matched experimental group was: FDR-0.0 = 5, FDR-20.0 = 7, Sated-0.0 = 6, Sated-20.0 = 7. On test day, average body weights of the Sated group was statistically significantly greater than the FDR group's body weight, t (23) = -10.12, $p < .001$, (see Figure 2). The mean number of active lever responses performed by the rats in the FDR group was almost three times higher than in the Sated group. The robust effect was confirmed by a statistically significant main effect of *feeding condition*, F (1,21) = 6.91, $p = .016$ (see Figure 3). The effect of the treatment with the CRF_1 receptor antagonist, R121919, on the number of active lever responses in the FDR and Sated groups is depicted in Figure 4. No statistically significant effects were found for *antagonist dose* or for the interaction *feeding condition* X *antagonist dose*. No statistically significant effects were observed for inactive lever responding on test day (see Figure 3 & 5).

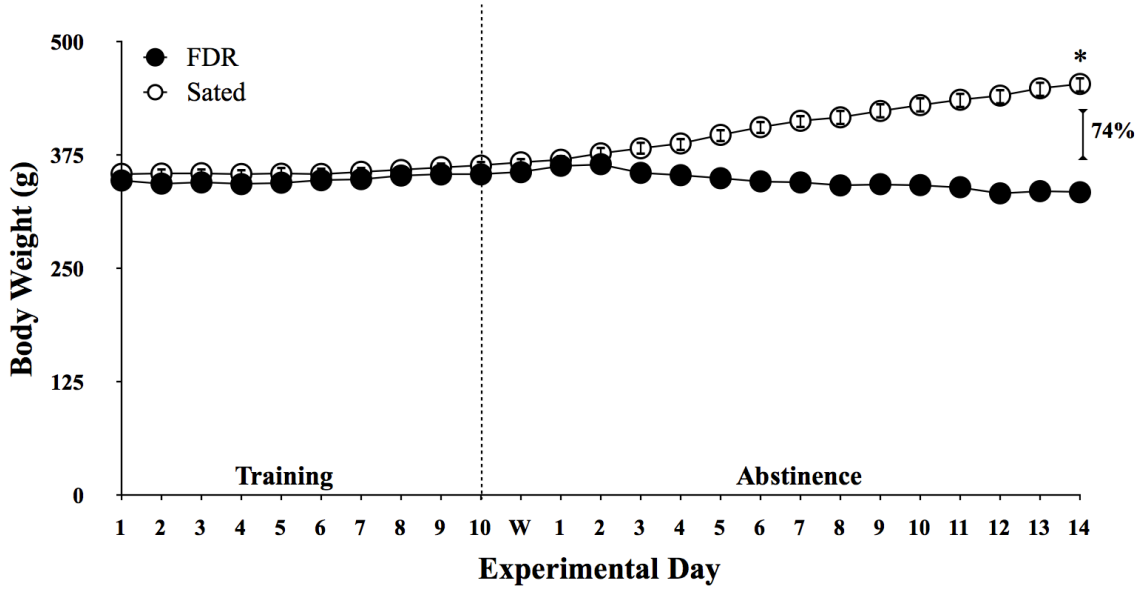

Figure 2. Mean (± SEM) body weights in the food restricted FDR (n = 12) and Sated (n = 13) groups, across experimental days for experiment 1A. All rats underwent 10 days heroin self-administration training in operant training chambers and 1 day drug washout in the animal care facility. Rats were matched according to body weight, active lever responding and drug infusions before separation into the FDR or Sated group. On day 14 of abstinence, rats were returned to the operant training chambers for a 1 h test under extinction conditions. * $p < .001$.

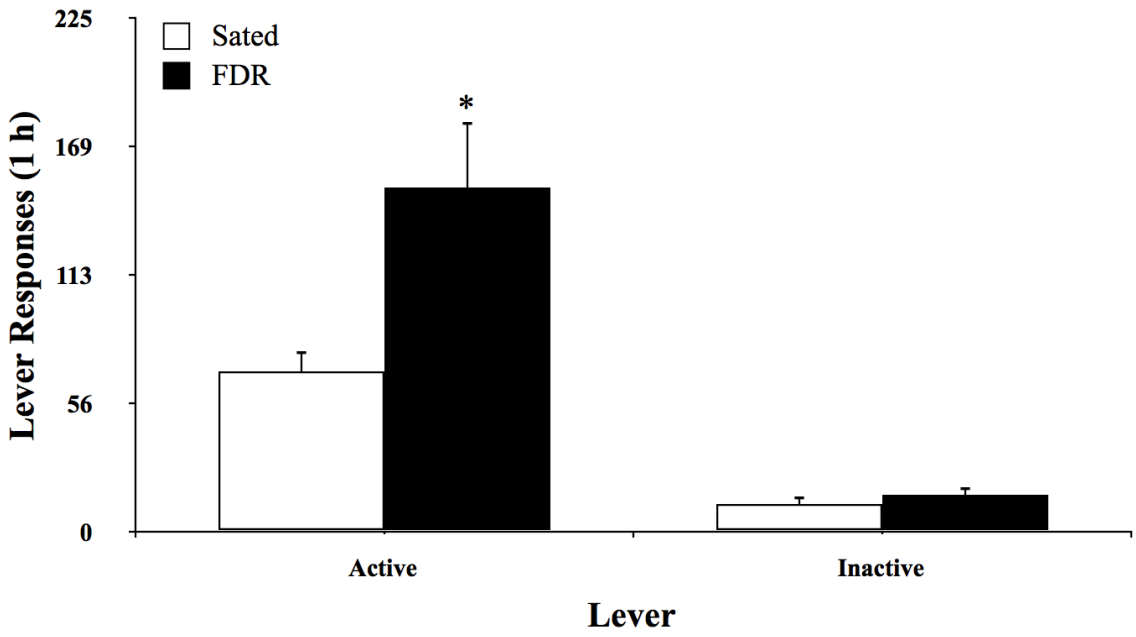

Figure 3. The effect of exposure to prolonged food restriction (FDR) on heroin seeking in abstinent rats. Data shown are the mean (+ SEM) active and inactive lever responding on test day, collapsed over FDR (n = 12) and Sated groups (n = 13) for experiment 1A. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. Active lever responding was statistically significantly greater for the FDR group versus Sated controls. * p = .016.

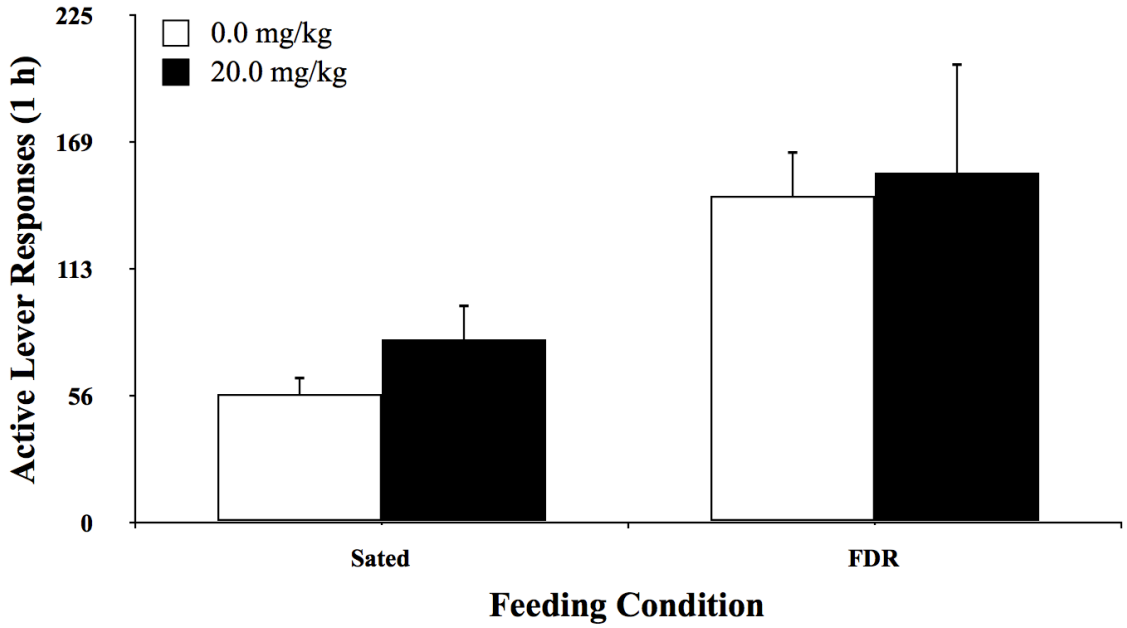

Figure 4. The effect of treatment with R121919 on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+ SEM) active lever responding on test day, in experiment 1A. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 20.0 mg/kg) in the FDR (*n's*: *FDR-0.0* = 6, *FDR-20.0* = 7) or Sated (*n's*: *Sated-0.0* = 5, *Sated-20.0* = 7) groups.

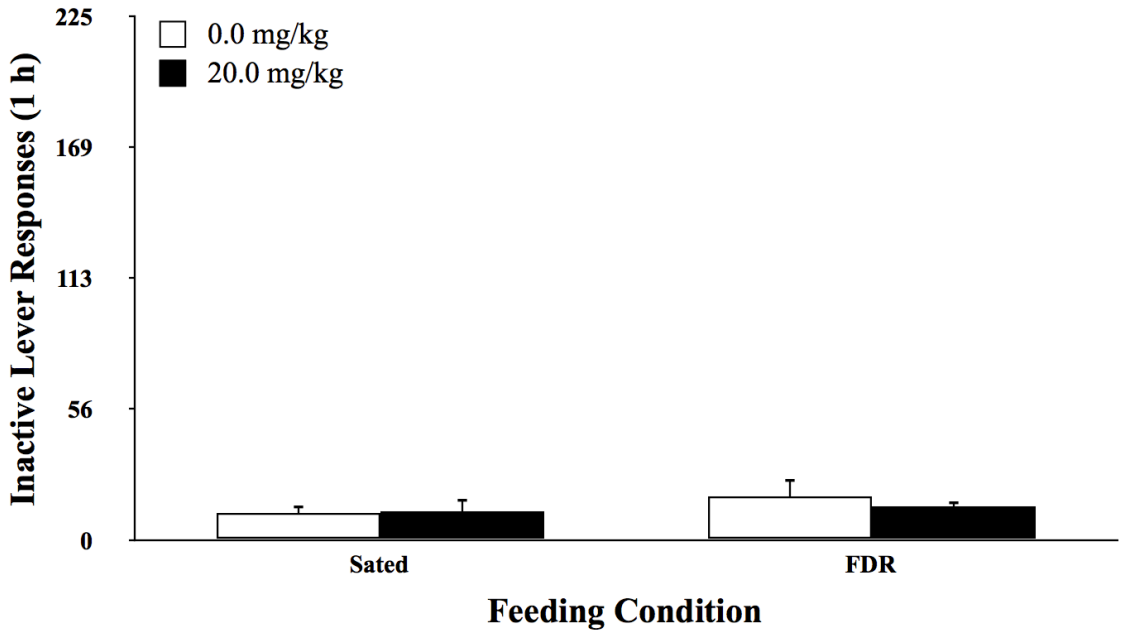

Figure 5. The effect of treatment with R121919 on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+SEM) inactive lever responding on test day, in experiment 1A. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 20.0 mg/kg) in the FDR (*n's*: *FDR-0.0* = 6, *FDR-20.0* = 7) or Sated (*n's*: *Sated-0.0* = 5, *Sated-20.0* = 7) groups.

Experiment 1B - The Effects of Treatment with the Non-selective CRF Receptor Antagonist, α-Helical CRF, on Chronic Food Restriction-induced Augmentation of Heroin Seeking in the Rat

Final analysis included 52 rats. A total of 8 rats were removed due to catheter leakage, failure to train or detached head-caps. The remaining rats acquired reliable heroin self-administration behavior.

Training. Mauchly's test of sphericity assumptions were violated for all training data. All values recorded were corrected using the Greenhouse-Geisser correction. A statistically significant increase in heroin infusions over time was observed, F (9,459) = 25.14, $p <$ .001. Active lever responding increased across training sessions, F (9,459) = 12.52, $p <$ .001, while no statistically significant change in inactive lever responding was found. Following the heroin self-administration phase, rats were separated into two groups FDR (n=26) or Sated (n=26) that were matched according to mean number of infusions, active lever responding and body weights over the last five days of training. Data on the last day of training is as follows; mean number of infusions (M = 35.06, SEM = 1.91), active and (M = 99.62, SEM = 13.40) inactive lever (M = 6.90, SEM = 0.84) responses.

Test. The number of rats in each matched experimental group was: FDR-0.0 = 8, FDR-10.0 = 11, FDR-25.0 = 7, Sated-0.0 = 10, Sated-10.0 = 11, Sated-25.0 = 5. On test day, average body weights of the Sated group (n = 26, M = 439.62, SEM = 9.27) were statistically significantly greater than the FDR groups (n = 26, M = 333.08 SEM = 4.17) body weights, t (50) = -10.48, $p <$ .001. Rats in the FDR group made a higher number of

responses on the active lever during the test, compared to the Sated group. This finding was supported by a statistically significant main effect of *feeding condition*, F (1,46) = 17.68, $p < .001$ (see Figure 6). Figure 7 shows effects of the treatment with the non-selective CRF receptor antagonist, *α-Helical CRF*, on the number of active lever responses in the FDR and Sated groups. No statistically significant effects were found for *antagonist dose* or for the interaction *feeding condition* X *antagonist dose*. No significant effects were observed for inactive lever responding on test day (see Figure 6 & 8).

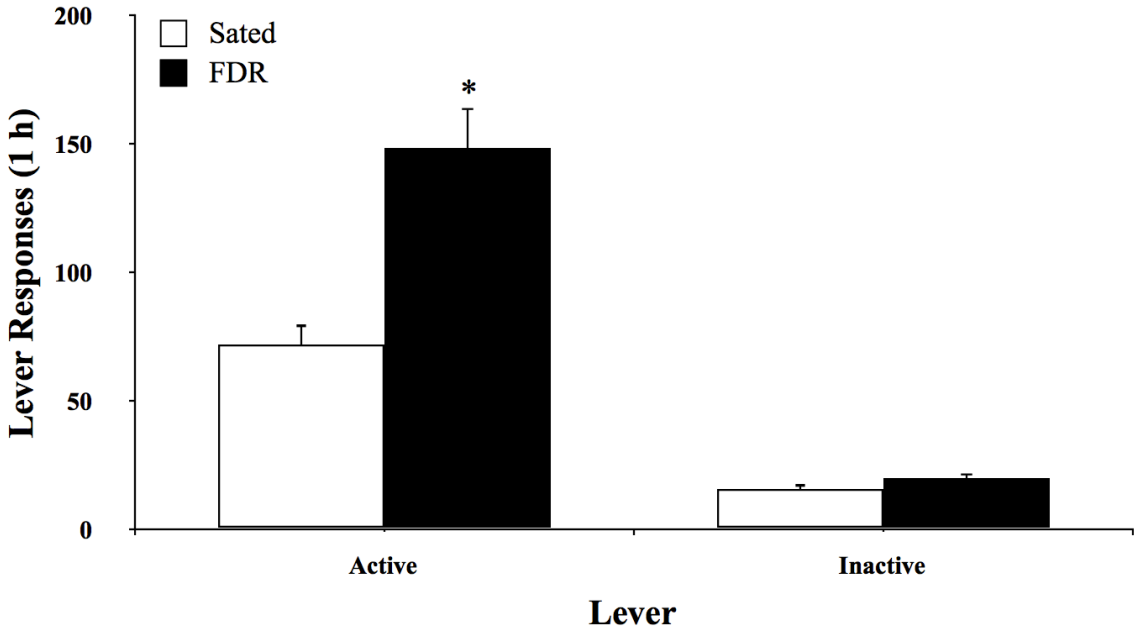

Figure 6. The effect of exposure to prolonged food restriction (FDR) on heroin seeking in abstinent rats. Data shown are the mean (+SEM) active and inactive lever responding on test day, collapsed over FDR (n = 26) and Sated groups (n = 26) for experiment 1B. Test day consisted of one-1 h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. Active lever responding was statistically significantly greater for the FDR group versus Sated control. * $p < .001$.

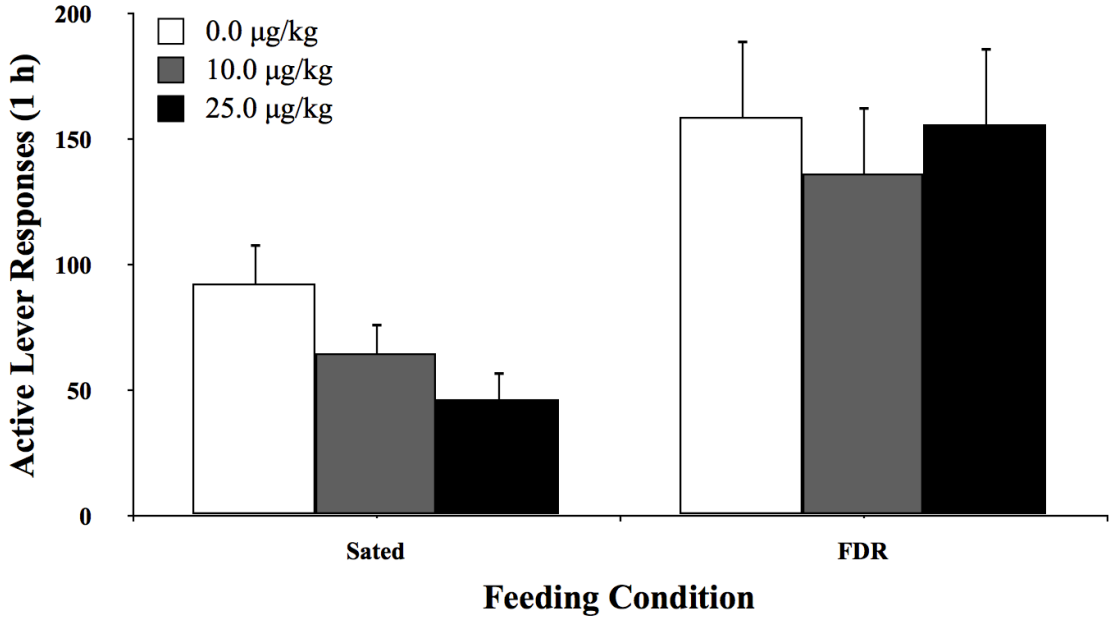

Figure 7. The effect of treatment with α-Helical CRF on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+ SEM) active lever responding on test day, in experiment 1B. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 10.0, 25.0 µg/kg) in the FDR (*n's*: *FDR-0.0* = 8, *FDR-10.0* = 11, *FDR-25.0* = 7) or Sated (*n's*: *Sated-0.0* = 10, *Sated-20.0* = 11, *Sated-20.0* = 5) groups.

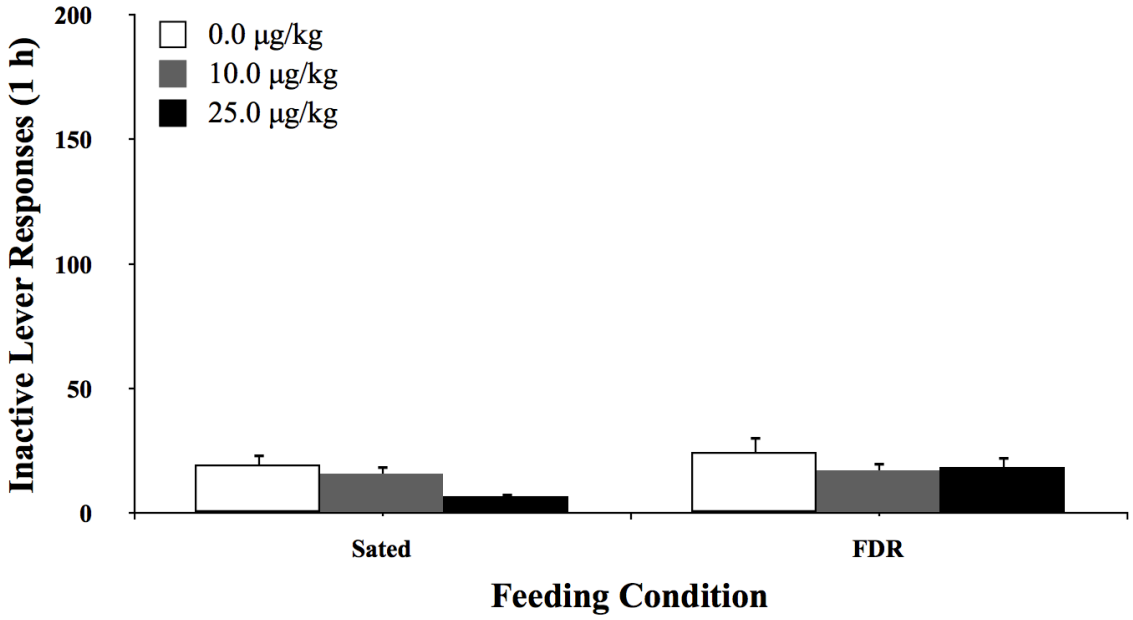

Figure 8. The effect of treatment with α-Helical CRF on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+SEM) inactive lever responding on test day, in experiment 1B. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 10.0, 25.0 µg/kg) in the FDR (*n's*: *FDR-0.0* = 8, *FDR-10.0* = 11, *FDR-25.0* = 7) or Sated (*n's*: *Sated-0.0* = 10, *Sated-20.0* = 11, *Sated-20.0* = 5) groups.

Anxiety Test. An independent samples t-test revealed a statistically significant decrease in latency to first consumption in the α-Helical CRF group versus vehicle controls, t (5) = 2.80, p = .038 (see Figure 9). A similar pattern was observed for number of approaches prior to first consumption, t (5) = 3.13, p = .021 (see Figure 10).

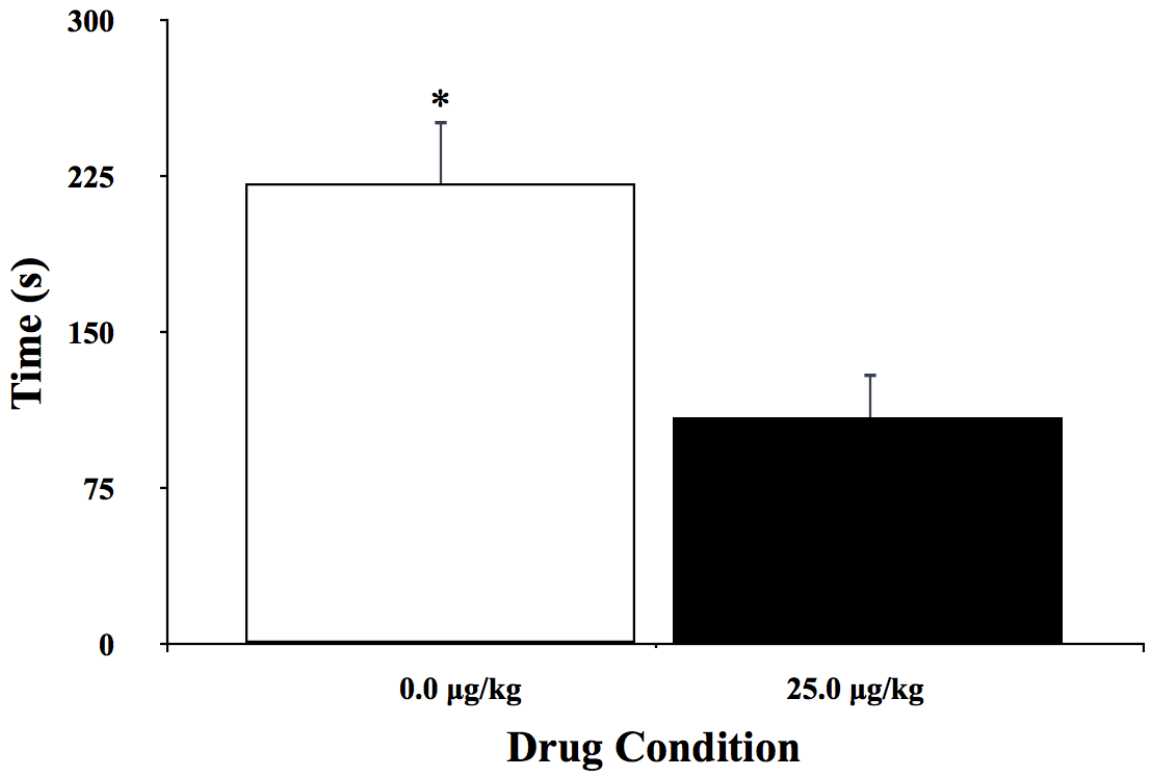

Figure 9. The effect of treatment with α-Helical CRF on anxiety measures in an open arena test in experiment 1B, following an 8 day food restriction period. Data shown are the mean (+ SEM) latencies to first consumption of a food pellet placed at the center of an open arena following injections of α-Helical CRF (0.0, 25.0 µg/kg; ICV), in food restricted rats (*n's*: *0.0 µg/kg* = 4, *25.0 µg/kg* = 3). * p = .038.

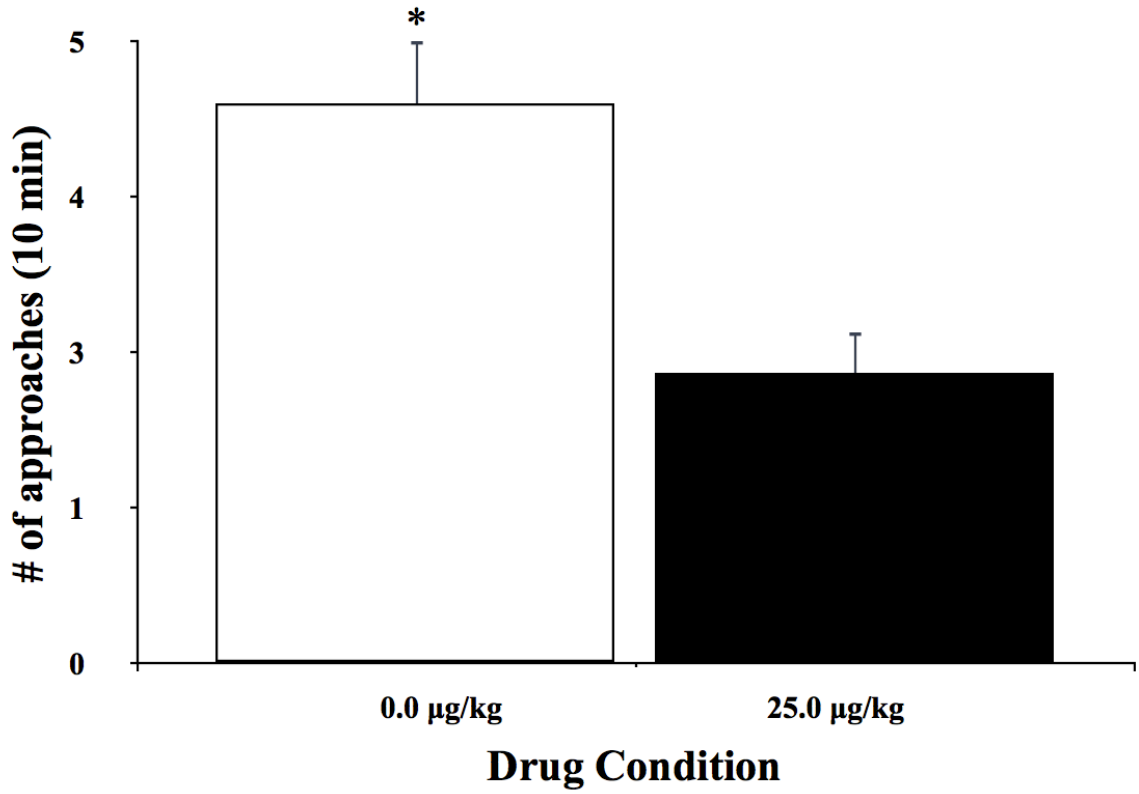

Figure 10. The effect of treatment with α-Helical CRF on anxiety measures in an open arena test in experiment 1B, following an 8 day food restriction period. Data shown are the mean (+ SEM) number of approaches to a food pellet placed in the center of an open arena prior to first consumption, following treatment with α-Helical CRF (0.0, 25.0 µg/kg; ICV), in food restricted rats (*n's*: *0.0 µg/kg* = 4, *25.0 µg/kg* = 3). * p = .021.

Experiment 2: The role of corticosterone in chronic food restriction-induced augmentation of heroin seeking in the rat.

Methods

Subjects

Twenty-seven male, Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 300-350g) were used. Rats were housed and treated as described in the general methods.

Surgical procedures

Rats were implanted with IV silastic catheters to allow for drug self-administration, as described in the general methods. Catheters were flushed daily with heparin/gentamicin to prevent blockage and infection.

Apparatus

The apparatus used was identical to that described in the general methods.

Drug

Heroin HCl was prepared as described in the general methods. RU486, a glucocorticoid receptor antagonist, was dissolved using a 25% β-Cyclodextrin (Sigma-Aldrich), 20% Dimethyl Sulfoxide (Fisher Scientific), 1% tween® 80 (INFO) and sterile water mixture. RU486 was adjusted to a pH of approximately 5.6 and injected intra peritoneal (IP) at a dose of 30 mg/kg.

Procedure

Self-administration training and abstinence phases procedures were identical to those described in the general methods.

Test Phase. On the morning of abstinence day 14, rats were given IP injections of RU486 (30.0 mg/kg) or vehicle. Rats were returned to the operant conditioning chambers and attached to the metal spring 45 min following the IP injections.

Plasma Corticosterone Determination

Immediately following the test phase (10h30), tail blood was collected, plasma was separated by centrifuge (Microlite RF Microcentrifuge, Thermo Fisher Scientific, Nepean, ON, Canada) at 10,000 rpm for 10 min. Samples were stored at -80°C. Plasma samples were analyzed for corticosterone levels using a corticosterone specific enzyme-linked immunosorbent assay (ELIZA) kit (Enzo Life Sciences: Cedarlane, Burlington, ON, Canada).

Statistical Analysis

All analysis were conducted using SPSS software (IBM, SPSS Statistics, version 20). Training data for all rats were analyzed using a within subjects ANOVA, with *training day* (1-10) as the independent variable and active lever responses, inactive lever responses or number of infusions as the dependent variable.

Number of responses on the active and inactive levers during the test session were analyzed using a univariate ANOVA. *Antagonist dose* (0.0, 30.0 mg/kg) and *feeding condition* (FDR, Sated) served as the independent variables.

Plasma corticosterone levels (pg/ml), sampled following the test session, were analyzed using a univariate ANOVA. *Antagonist dose* (0.0, 30.0 mg/kg) and *feeding condition* (FDR, Sated) served as the independent variables. A statistically significant

interaction was followed by appropriate post hoc tests. The critical cut of point for statistically significant results was $p \leq .05$.

Results

Final analysis included 27 rats. Three rats were removed due to catheter leakage, failure to train or detached head-caps. The remaining rats acquired reliable heroin self-administration behavior.

Training. Mauchly's test of sphericity assumptions were violated for all training data. All values recorded were corrected using the Greenhouse-Geisser correction. A statistically significant increase in heroin infusions over time was observed, F (9,234) = 14.38, $p < .001$. Active lever responding increased across training sessions, F (9,234) = 12.16, $p < .001$, while no statistically significant change in inactive lever responding was observed. Data on the last day of training is as follows: mean number of infusions (M = 39.89, SEM = 4.44), active and (M = 141.11, SEM = 22.57) inactive lever (M = 17.41, SEM = 7.98) responses.

Test. The number of rats in each matched experimental group was: FDR-0.0 = 7, FDR-30.0 = 7, Sated-0.0 = 8, Sated-30.0 = 5. On test day, average body weights of the Sated group (n = 13, M = 426.23, SEM = 28.93) were statistically significantly greater than the FDR group's (n =14 M = 317.36, SEM = 19.50) body weight, t (25) = -11.54, $p < .001$. As can be seen in Figure 11, the FDR group showed a statistically significant increase in active lever responding compared to the Sated group, (*feeding condition effect*: F (1,23) = 8.46, p =.008). No statistically significant effects for *antagonist dose* or the interaction

feeding condition X *antagonist dose* were observed, (see Figure 12). A statistically significant increase in inactive lever responding for the FDR group versus the Sated group was also found, F (1,23) = 6.42, p =.019 (see Figure 11). No other statistically significant effects were found for inactive lever responses (see Figure 13).

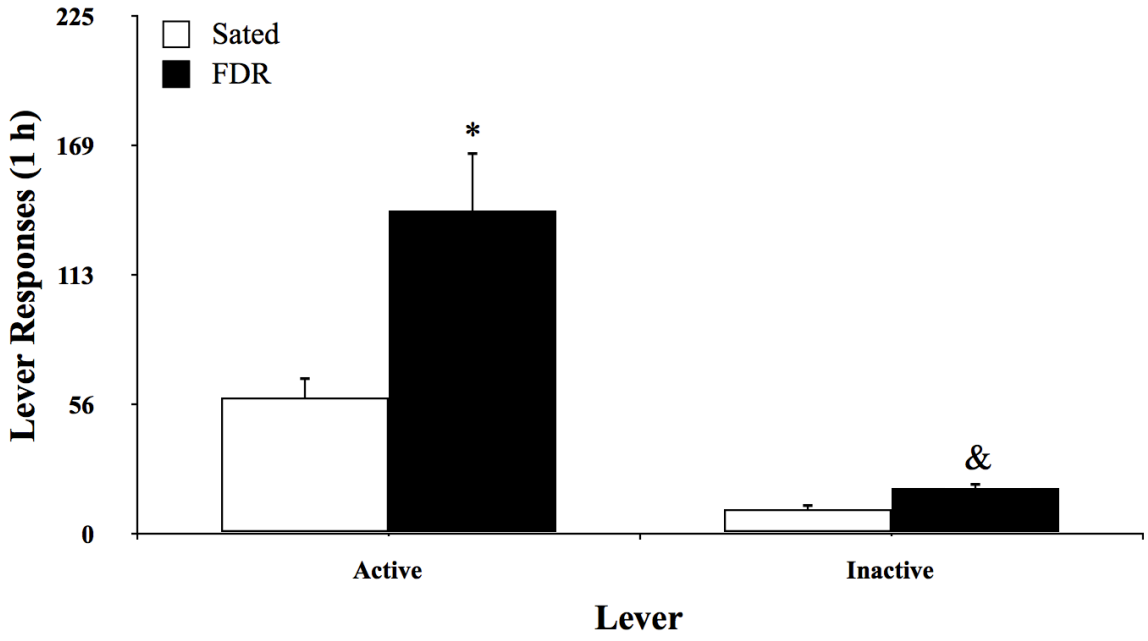

Figure 11. The effect of exposure to prolonged food restriction (FDR) on heroin seeking in abstinent rats. Data shown are the mean (+ SEM) active and inactive lever responding on test day, collapsed over FDR (n = 14) and Sated groups (n = 13) for experiment 2. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 16 days of abstinence under FDR or sated conditions. Active and inactive lever responding was statistically significantly greater for the FDR group versus Sated controls. * p = .008; $^{\&}$ p = .019.

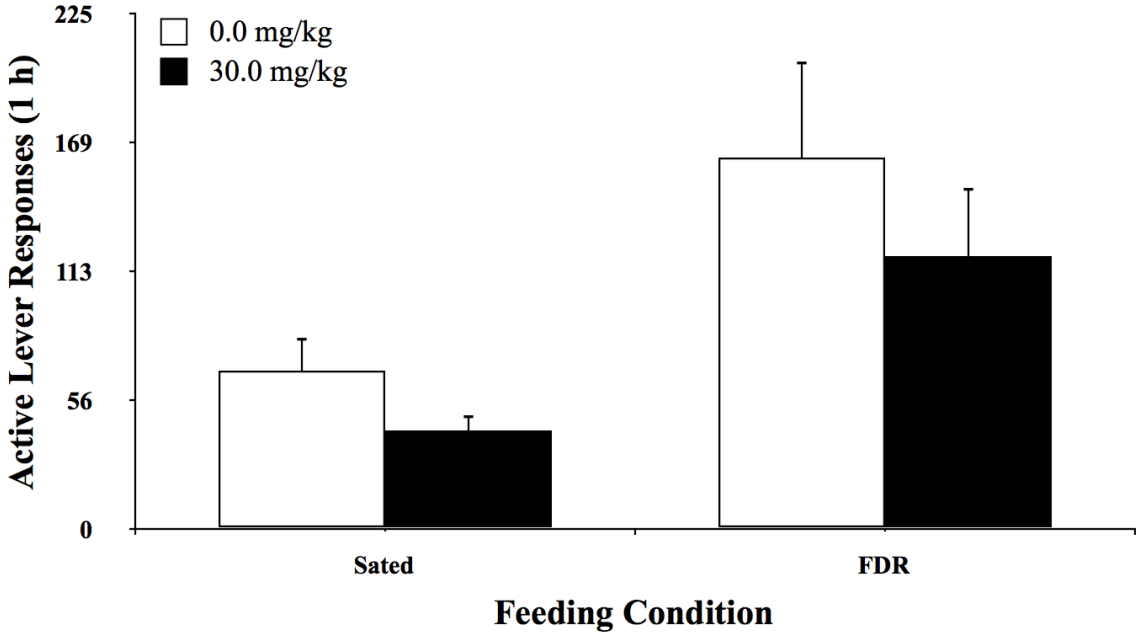

Figure 12. The effect of treatment with RU486 on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+SEM) active lever responding on test day, in experiment 2. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 16 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 30.0 mg/kg) in the FDR (*n's*: *FDR-0.0* = 7, *FDR-30.0* = 7) or Sated (*n's*: *Sated-0.0* = 8, *Sated-30.0* = 5) groups.

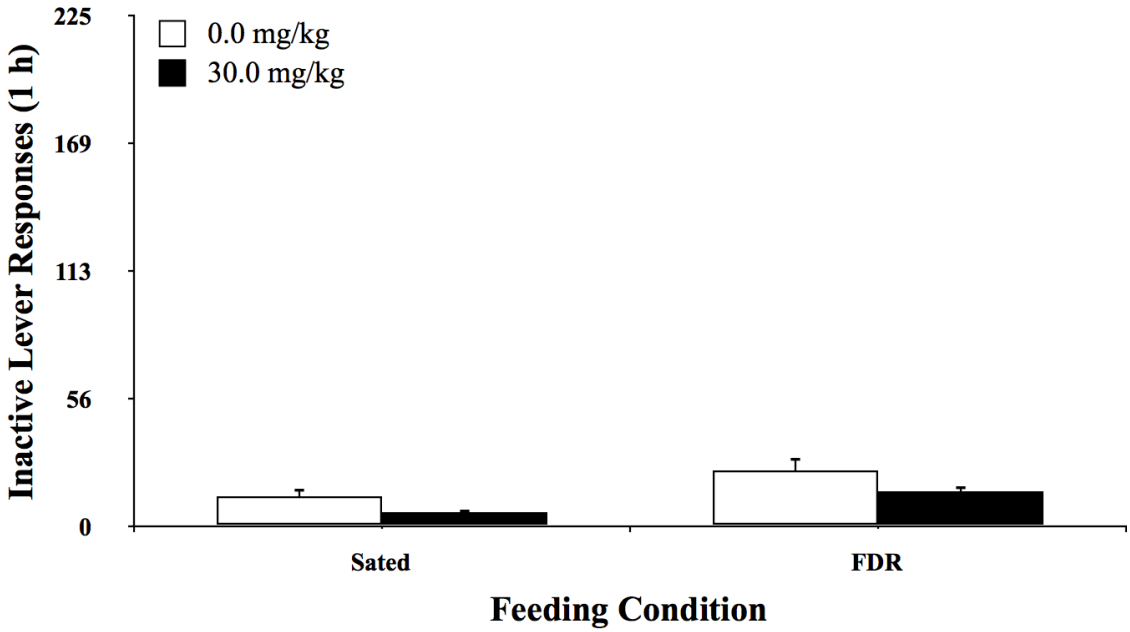

Figure 13. The effect of treatment with RU486 on heroin seeking in food restricted (FDR) and sated rats. Data shown are the mean (+SEM) inactive lever responding on test day, in experiment 2. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 16 days of abstinence under FDR or sated conditions. No statistically significant results were observed across antagonist dose (0.0, 30.0 mg/kg) in the FDR (*n's*: *FDR-0.0* = 7, *FDR-30.0* = 7) or Sated (*n's*: *Sated-0.0* = 8, *Sated-30.0* = 5) groups.

Plasma Corticosterone Determination. Analysis of plasma corticosterone levels taken immediately following the test session revealed a statistically significant interaction between *feeding condition* and *antagonist dose, F* (1, 15) = 7.15, $p = .017$. Independent samples *post-hoc* t-tests with a Bonferroni-adjusted $\alpha = .025$, revealed a statistically significant increase in corticosterone levels in rats treated with 30.0 mg/kg RU486 versus the 0.0 mg/kg dose for the FDR group, t (7) = -2.99, $p = .020$. A similar effect was observed in the Sated group, t (8) = -5.59, $p = .001$, (see Figure 14).

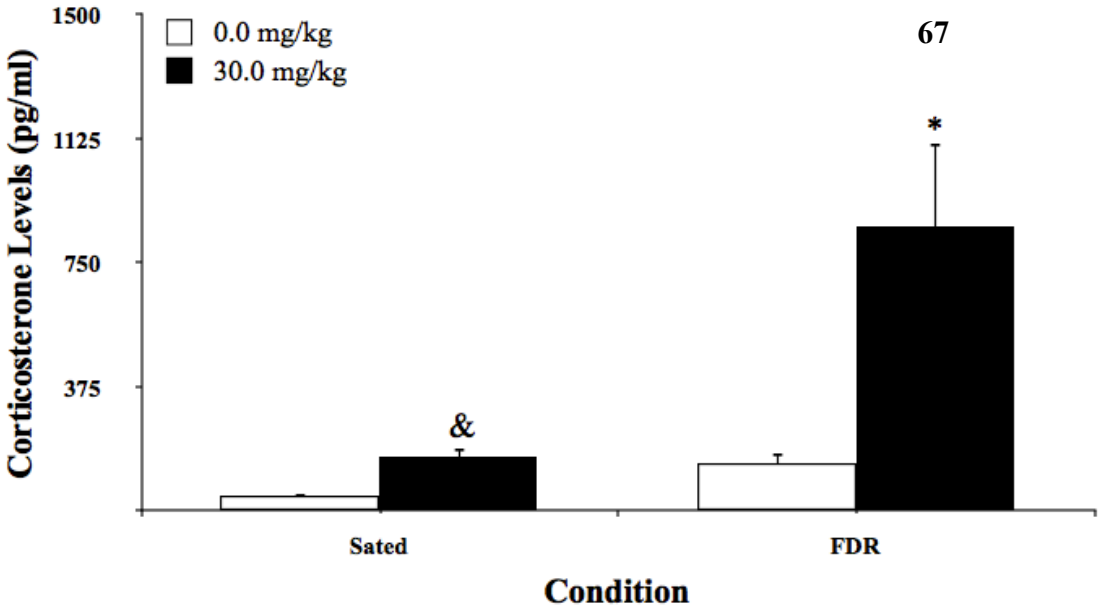

Figure 14. The effect of treatment with RU486 on corticosterone levels in experiment 2. Data shown are the mean (+SEM) corticosterone levels (pg/ml) immediately following a test session under extinction conditions after infusions of RU486 (0.0, 25.0 µg/kg; ICV), in food restricted rats (*n's*: *0.0* = 12, *30.0* = 7) and Sated rats (*n's*: *0.0* = 8, *30.0* = 5). * p = .020; $^{\&}p$ = .001.

Summary

A robust increase in heroin seeking was observed in food restricted, compared to sated rats, when animals were returned to the heroin self-administration environment. In contrast to our hypothesis, this augmentation in heroin seeking was not attenuated by treatment with a selective CRF_1 or non-selective CRF receptor antagonist. Additionally, glucocorticoid receptor antagonism had no effect on the augmentation of heroin seeking in food restricted, abstinent rats.

Consequently, the goal of the experiment in Chapter 2 was to investigate neuronal activation in brain areas implicated in reward-related processes and drug seeking. Identifying brain sites involved in the augmentation of heroin seeking following prolonged food restriction will offer neural targets for future studies, indented to elucidate the underlying mechanisms of our effect.

Chapter 2

The effects of chronic food restriction on Fos immunoreactivity in the nucleus accumbens in heroin seeking rats.

Sedki, F., D'Cunha, T., Awadallah, S., & Shalev, U.

Introduction

There is evidence that stress has an important influence on drug taking and drug reward (Carr, 2002; Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003a). Recently, we reported an augmentation of heroin seeking in abstinent, chronically food-restricted rats (D'cunha et al., 2012). However, this effect was not attenuated by acute manipulation of the corticotropin releasing factor (CRF) or corticosterone stress systems (Chapter 1). This indicates that an acute physiological stress-response is not critically involved in the augmentation of heroin seeking following prolonged food restriction. Here we present an exploratory study aimed at the identification of relevant brain sites, as the neural mechanisms mediating this effect remain elusive.

Drugs of abuse exert their effects by acting upon neuronal circuitry that evolved to encourage species survival such as feeding and mating (Balfour, Yu, & Coolen, 2004; Glickman & Schiff, 1967; Kelley & Berridge, 2002; Wise & Bozarth, 1985). The mesocorticolimbic dopamine (DA) circuit is strongly implicated in appetitive motivation and the learned aspects of addictive behavior, with considerable evidence supporting its involvement in the rewarding properties of natural stimuli and abused drugs (Berridge & Kringelbach, 2008; Feltenstein & See, 2008; R. Wise, 1996). Additionally, it is thought that stress has a modulatory role on this pathway. The mesocorticolimbic DA circuit contains cell bodies in the ventral tegmental area which project to limbic structures, such as the amygdala and nucleus accumbens (NAc), as well as cortical areas such as the medial prefrontal cortex (mPFC). Here, we chose to investigate brain sites that are involved in stress and reward related processes; the amygdala, nucleus accumbens (NAc), and medial prefrontal cortex (mPFC).

Importantly, the amygdala exhibits increased levels of CRF (Cook, 2004) following exposure to stress. Furthermore, disconnecting CRF-containing projections from the central nucleus of the amygdala (CeA) to the bed nucleus of the stria terminalis (BNST), results in a blockade of footshock stress-induced reinstatement of cocaine seeking (Erb et al., 2001). Together, these studies support the notion that the amygdala is involved in stress responsivity, as well as the motivational processing of conditioned drug rewards. McFarland and colleagues (2004) provide further evidence that the amygdala is involved in the modulation of conditioned drug rewards. For example, inhibition of the CeA via local injections of gamma aminobutyric acid (GABA) receptor agonists attenuated footshock-induced reinstatement of cocaine seeking. Another sub-region of the amygdala, the basolateral amygdala (BLA), mediates the effects of exposure to drug-associated cues on drug seeking. Transient inactivation of the BLA can block cue-induced reinstatement of cocaine and heroin seeking (Fuchs & See, 2002; Grimm & See, 2000), while excitotoxic lesions of the BLA can disrupt the incentive properties of conditioned stimuli (Meil & See, 1997; Whitelaw, Markou, Robbins, & Everitt, 1996). This suggests that while the primary reinforcing properties of the drug itself are unaffected by lesions of the BLA, the ability to acquire a learned behavior which is reinforcing as a result of its association with the drug, the primary reinforcer, (second-order conditioning) is disrupted (Meil & See, 1997; Whitelaw et al., 1996). Yun and colleagues (2003) demonstrated that in addition to the attenuation of cocaine-induced reinstatement, lesions of the BLA impair a rodents ability to distinguish cues which predict the availability of a cocaine reward. In contrast with the inactivation of the BLA, activation by means of a brief 20 Hz electrical stimulation or NMDA-induced chemical

stimulation results in the reinstatement of cocaine seeking (Hayes, Vorel, Spector, Liu, & Gardner, 2003). Additionally, heightened neuronal activation, assessed through the expression of the immediate early gene *c-fos*, in the BLA, is associated with cue-induced reinstatement of ethanol seeking (Jupp, Krstew, Dezsi, & Lawrence, 2011; Schroeder et al., 2008). While the BLA is critical to the acquisition of learned stimulus-reward associations, as well as the expression of cue-induced cocaine seeking, lesions of the CeA are instead implicated only in the expression, but not the acquisition of these behaviors (Kruzich & See, 2001). Taken together, these studies strongly implicate the amygdala in responding not to the drug rewards, but to acquisition of and responses to conditioned stimuli that were associated with drug rewards.

Studies have unequivocally demonstrated a role for the NAc in reward-related, goal-directed and drug seeking behaviors (Bossert et al., 2005; Carlezon & Thomas, 2009; Fuchs, Ramirez, & Bell, 2008b). Moreover, the finding that experiencing stress during development alters spine density, dendritic length, and branching in the NAc suggests a role for the NAc in stress control (Muhammad, Carroll, & Kolb, 2012). With respect to drug rewards, lesions of the NAc have been found to reduce the self-administration of cocaine, morphine and heroin (Dworkin, Guerin, Co, Goeders, & Smith, 1988; Zito, Vickers, & Roberts, 1985). Additionally, following inactivation of the NAc, a reduction in context- and priming-induced reinstatement of cocaine seeking is observed (Fuchs, Ramirez, & Bell, 2008b; Grimm & See, 2000). A reduction in cue-induced heroin seeking and ethanol renewal, i.e., the return to drug seeking in a previously drug paired context following extinction in a different context ethanol seeking,

has also been reported following transient inactivation of the NAc (Chaudhri, Sahuque, Schairer, & Janak, 2009; Rogers, Ghee, & See, 2008).

Two anatomically and functionally distinct sub-compartments of the NAc; the core (NAcC) and the shell (NAcS), have been identified. The NAcC is necessary for motor control and the expression of learned behaviors, while the NAcS is implicated in limbic aspects such as sensory, emotional control as well as ingestive behaviors (Kelley, 2004). These sub-regions seems to be critically involved in the reinstatement of extinguished drug seeking. Thus, blockade of DA D1-receptors in the NAcS, or interfering with glutamate release in this area by injection of an mGluR2/3 agonist, attenuates context-induced reinstatement of heroin seeking (Bossert, Poles, Sheffler-Collins, & Ghitza, 2006; Bossert, Poles, Wihbey, Koya, & Shaham, 2007). Additionally, antagonizing mGluR1 or AMPA/kainate receptors in the NAcC impairs the expression of context-induced cocaine seeking in rats (Xie et al., 2011), and an increase in glutamate release in the NAcC is required for the reinstatement of cue-induced heroin seeking (LaLumiere & Kalivas, 2008). Finally, the blockade of DA D1-receptors in the NAcC attenuated the reinstatement of discrete cue-, but not context-induced heroin seeking (Bossert et al., 2007). Differential roles for these sub-regions was also reported with ethanol seeking, where inactivation of the NAcS, but not the NAcC attenuated contextual renewal of ethanol seeking (Chaudhri et al., 2009). An important role has been suggested for the NAc in stress-induced reinstatement as well, as inactivation of the NAcS or NAcC can inhibit footshock-induced reinstatement (McFarland et al., 2004).

Finally, the prefrontal cortex (PFC) is known to be important for reward seeking. Generally, the prefrontal cortex is linked to executive functioning (Rossi, Pessoa,

Desimone, & Ungerleider, 2009). This includes the organization of thoughts and actions necessary for goal-related behaviors, the regulation of appetite, and more specifically the acquisition of instrumental learning (Fregni et al., 2008; McLaughlin & See, 2003). Furthermore, as a result of repeated exposure to cocaine, adaptations in dendritic spine shape are reported in the PFC (Robinson, Gorny, Mitton, & Kolb, 2001). These region-specific alterations may contribute to dysfunctions in executive control and decision making, a hallmark of drug addiction.

Several sub-sections of the PFC have been implicated in drug reward-related processes. The prelimbic (pl-PFC) and infralimbic (il-PFC) cortices appear to mediate different aspects of behavior; the il-PFC is involved in habitual, persistent behaviors and the pl-PFC is involved in the acquisition of drug seeking behavior and the reacquisition of drug-taking after extinction (Di Ciano, Benham-Hermetz, Fogg, & Osborne, 2007). For example, an attenuation of discrete cue-, cocaine-priming, and stress-induced reinstatement is achieved through inactivation of the pl-PFC, but not the il-PFC (Capriles et al., 2003; McFarland & Kalivas, 2001; J. McLaughlin & See, 2003). The attenuation of context-induced reinstatement, however, was demonstrated by inactivation of the il-PFC (Bossert et al., 2011). The pl-PFC is implicated in stress-induced reinstatement, as greater activation in this region is observed following acute-food deprivation induced heroin seeking (Shalev, Robarts, Shaham, & Morales, 2003b). In a conditioned place preference paradigm, however, rats with a greater preference for the cocaine paired context had reduced levels of neural activation of the pl-PFC when compared to low preference or non-drug conditioned control rats (Zombeck et al., 2008).

The findings described above suggest that the mesocorticolimbic DA circuitry, including the amygdala, NAc, and mPFC is critically involved in drug and non-drug reward seeking. Thus, the goal of the present study, was to investigate whether these brain sites that are potentially involved in food restriction-induced augmentation of heroin seeking in abstinent rats.

To that end, we used Fos protein immunoreactivity (Fos-IR) to identify neurons that are activated by re-exposure to the drug self-administration context and cues in food restricted and sated rats, during tests for heroin seeking following prolonged abstinence. The immunohistochemical localization of Fos (the protein product of the immediate early gene *c-fos*) is commonly used to study neuronal activity following environmental and pharmacological manipulations (Morgan & Curran, 1991). Fos-IR was previously used for the identification of brain sites that are involved in cue, or stress-induced reinstatement of drug seeking (Neisewander, O'Dell, Tran-Nguyen, Castañeda, & Fuchs, 1996; Shalev, Robarts, Shaham, & Morales, 2003b), and in chronic food restriction-induced increases in behavioral responsiveness to drugs of abuse (Carr, 2002).

Four control groups were included in the current study. Fos-IR was determined in groups of drug-naive rats under sated or food restricted conditions to assess the effects of food restriction per se on neuronal activation. In addition, since active extinction of drug seeking alters brain adaptations induced by previous exposure to drugs (Self & Choi, 2004; Sutton et al., 2003), we also assessed neuronal activation in heroin-trained sated or food restricted rats that were not exposed to the drug-seeking test session.

Fos-IR was examined in the BLA, CeA, NAcS and NAcC, the pl-PFC and il-PFC, all brain sites that are involved in stress and reward. It was expected that, across all

experimental conditions, exposure to a prolonged period of food restriction would result in increased activation compared to rats allowed unrestricted access to chow, and that Fos-IR would be highest in the heroin-trained food restricted rats that were exposed to the drug-seeking test.

Methods

Subjects

Forty-three male Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 300-350g) were used. Rats were housed under conditions specified in the general methods section.

Surgical procedures

As described in the general methods, rats were implanted with IV silastic catheters to allow for drug self-administration. Catheters were flushed daily with heparin/gentamicin to prevent blockage and infection.

Apparatus

Operant conditioning chambers. Heroin training was conducted in operant-training chambers identical to those described in the general methods.

Drug

Heroin HCl was prepared as described in the general methods.

Procedure

The experiment included 3 major groups of rats. The first, heroin-trained-tested group (n=25), was exposed to the heroin self-administration training, abstinence, and test phases as described in the general methods. The second, heroin-trained-no-test group

(n=8), was treated similarly to the first group, except that the rats were sacrificed without being tested for drug seeking at the end of the abstinence period. Rats in the third, drug-naive group (n=10), remained in the ACF for the entirety of the experiment. Each group was further divided into food restricted (FDR) and sated sub-groups. Food restricted rats were restricted throughout the abstinence phase (or a 14-day period for the naive rats), as described in the general methods, aiming to keep their mean body weight at 80% of the sated rats'. Rats were sacrificed immediately after their test session (heroin-trained-tested group) or at the approximate time the test session would have ended (heroin-trained-no-test and drug-naive groups), at approximately 10:30 AM.

Immunohistochemistry

Following the test session, rats were injected with Euthanyl (Sodium Pentobarbital, 64.8mg/ml), transcardially perfused using 4% paraformaldehyde and phosphate buffered saline (NaH2PO4; sodium phosphate monobasic, NaOH; salt pellets, distilled water) and then decapitated. Brains were extracted, preserved in a solution of 4% paraformaldehyde for 48 h and then sliced to obtain 40 μm sections on a vibratome 3000 Deluxe (Harvard Apparatus Canada, Saint-laurent, QC, CA). Tissue was sliced and collected according to coordinates provided by the Paxinos & Watson (2005) atlas. Nucleus accumbens (NAc) tissue slices ranged from 3.24 to 1.20 in relation to bregma. Amygdala tissue slices ranged from -1.56 to -3.48 in relation to bregma. Tissue sections were washed used Trizma Buffered Saline and placed in 4°C for 90 min in a solution of Trizma Buffered Saline/Milk Buffer (TBS-M), Triton X-100 and Normal Goat Serum (NGS). Tissue sections were then placed in a solution of NGS, TBS, Tritron X-100 and the primary antibody (rabbit anti-c-Fos antibody: Calbiochem, San Diego, CA) at 4°C,

for 48 h. Tissue sections were washed in TBS, and placed in a mixture of hydrogen peroxide (H_2O_2) and TBS for 30 min in 4°C to reduce nonspecific binding. Next, sections were washed in TBS and incubated in a solution of TBS, Biolinylated Anti-rabbit IgG (secondary antibody), NGS and Triton X-100 at 4°C. After rinsing with TBS, sections were incubated in avidin-biotinylated horseradish peroxidase (Vectastain ABC kit, Vector Laboratories, Burlington, ON, CA). Slices were then rinsed in TBS and developed in a solution of 3, 3-diaminobnzidine-4 HCl (DAB), H_2O_2 and Nickel Chloride for 1 min and 30 sec. Slices were mounted on slides, air dried, dehydrated with ethanol, and cover slipped.

Image Analysis

Immunolabeled sections were examined using a Leica DMLA Microscope (Leica Microsystems, Germany) and a Qimaging Fast 1394 Camera (Surrey, BC). Image J (Wayne Rasband, National Institude of Health, Bethesda, Maryland, U.S.) was used for capturing brain images at 10x magnification and quantifying Fos protein immunoreactivity. A built in *Yen* protocol was used to quantify cell counts. Cell Circularity (.3-1) and cell size (30-150 pixels^2) were adjusted for greater accuracy. The brain regions of interest were identified based on the Paximus and Watson atlas (2005). To account for variability in region size, each relevant brain region was manually traced and its area determined by the software. *c-Fos* immunoreactivity (IR)-labeled nuclei counts were divided by their respective areas and multiplied by 10^6. The 6 brain slices representing the highest Fos protein IR in each brain for each rat area were chosen for statistical analysis (see Figure 15). The means of these top 6 slices for each rat were used in the final analysis.

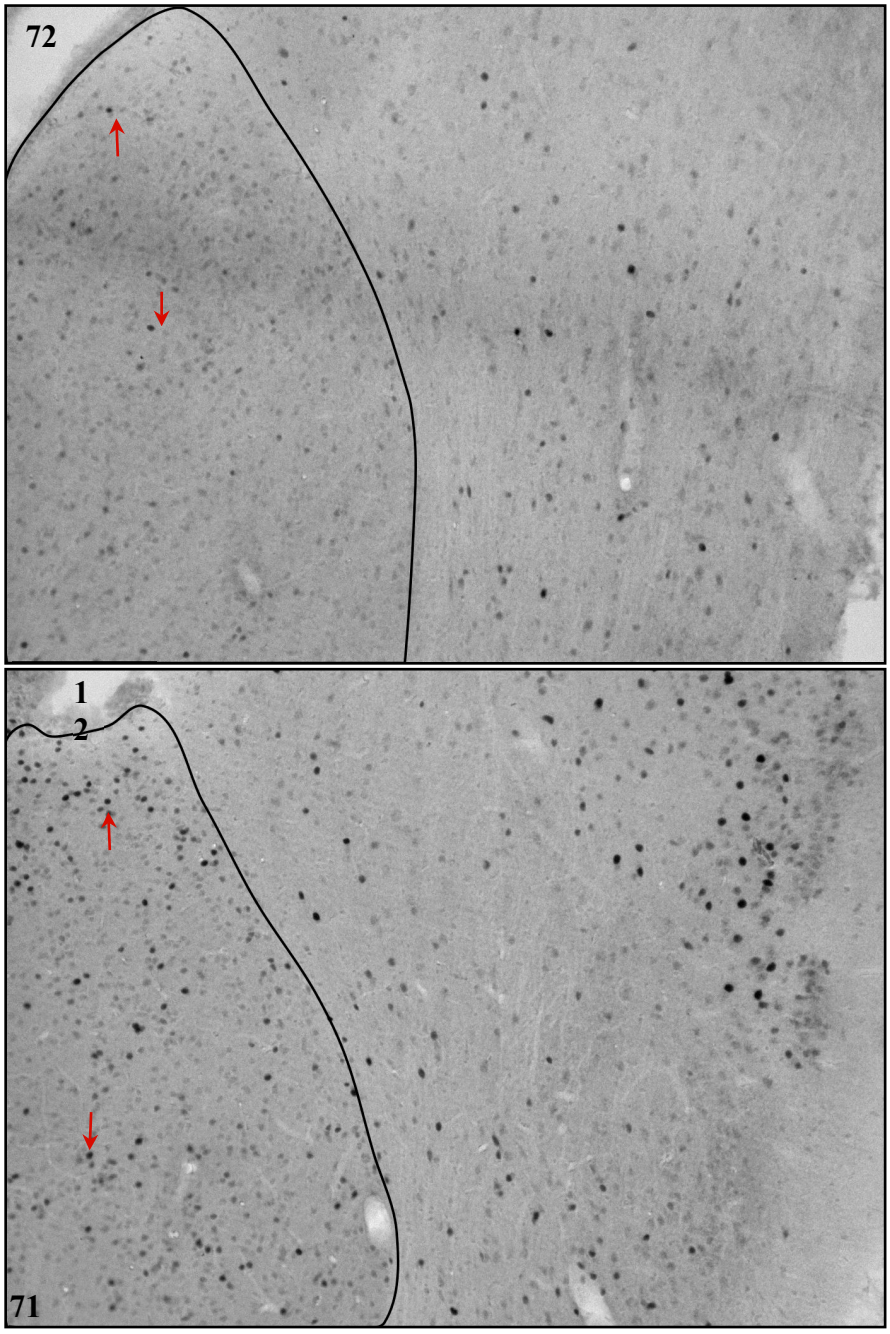

Figure 15. Fos protein IR-labeling in the nucleus accumbens shell (NAcShell) and ventricles (V) in the heroin-trained-tested condition. **A** (upper panel): pictograph of representative tissue slice from the food restricted (FDR) group (n = 11). **B** (lower panel): pictograph of representative tissue slice from the Sated group (n = 14). Following self-administration training, on abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions.

Statistical Analyses

All analysis were conducted using SPSS software (IBM, SPSS Statistics, version 20). Training data for the heroin-trained tested group and the heroin-trained-abstinence group were analyzed using a within subjects ANOVA, with *training day* (1-10) as the independent variable and active lever responses, inactive lever responses or number of infusions as the dependent variable.

Number of responses on the active and inactive levers for the heroin-trained-tested group during the test session were analyzed using an independent samples t-test with *feeding condition* (FDR, Sated) as the independent variables.

Three *a priori*, independent samples t-tests were carried out for each group (heroin-trained-tested, heroin-trained-no-test, drug-naive), across all brain region (NAcShell, NAcCore, basolateral amygdala (BLA) and central nucleus of the amygdala (CeA)) to compare the level of Fos protein IR. *Feeding condition* (FDR, Sated) served as the independent variable while *Fos levels* adjusted for region size served as the dependent variable. The critical cut-off point for statistically significant results was $p \leq 0.05$, with the exception of the Fos protein comparisons, where analysis in each brain region used a Bonferronni adjusted alpha level of $\alpha = .017$.

Results

Final analysis included a total of 43 rats. (heroin-trained-tested, $n = 25$; heroin-trained-no-tests $n = 8$; drug-naive, $n = 10$). Seven rats were removed due to catheter leakage, failure to train or issues during the perfusion process. The remaining rats acquired reliable heroin self-administration behavior.

Behavioral Analysis

Heroin-trained-tested group

Training. Mauchly's test of sphericity assumptions were violated for all training data. All values recorded were corrected using the Greenhouse-Geisser correction. A statistically significant increase in heroin infusions over time was observed, F (9,270) = 12.70, p < .001. Active lever responding increased across training sessions F (9,270) = 5.67, p = .006, while no statistically significant change in inactive lever responding was found. Since some rats were removed from the analysis after the group matching was conducted (due to inadequate brain perfusion), last five days of training data was used to compare the rats assigned to the FDR (n=11) and Sated (n=14) groups. No statistically significant differences were found across all criteria: number of infusions, active lever responding and body weights.

Test. On test day, average body weights of the Sated group (M = 499.92, SEM = 34.85) were statistically significantly greater than the FDR groups (M = 335.18, SEM = 16.95) body weights, t (22) = -10.81, p < .001. Furthermore, the FDR group showed a statistically significant increase in active lever responding compared to the Sated group, t (23) = 5.21, p < .001 (see Figure 16). No statistically significant difference was observed for inactive lever responding (see Figure 16).

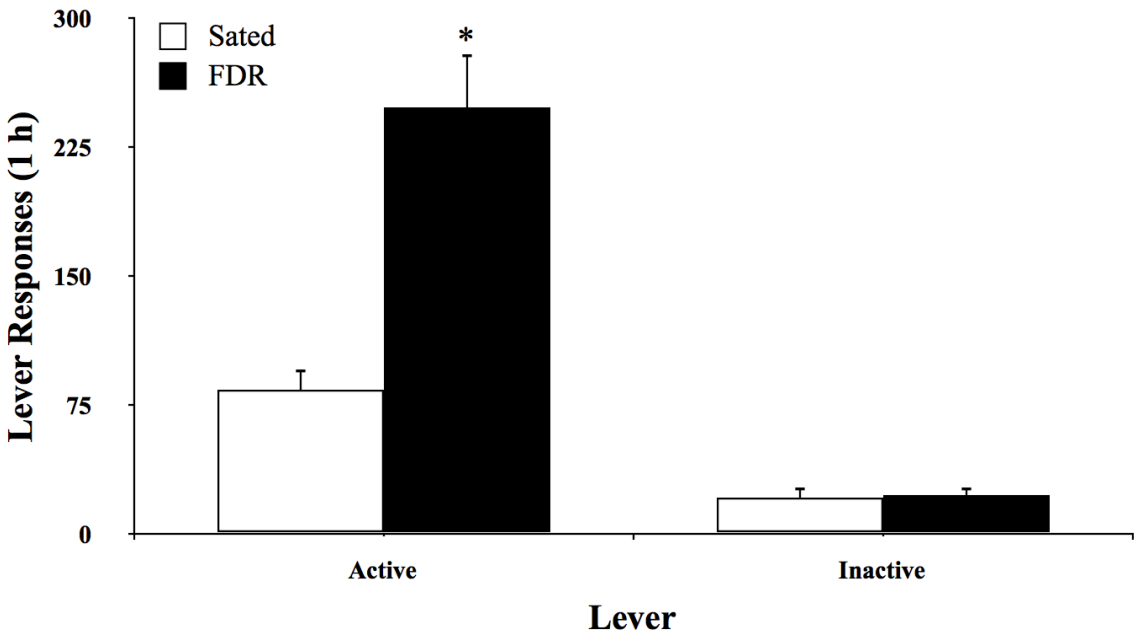

Figure 16. The effect of exposure to prolonged food restriction (FDR) on heroin seeking in abstinent rats. Data shown are the mean (+SEM) active and inactive lever responding on test day, for the FDR (n =11) and Sated groups (n = 14) for experiment one. Test day consisted of one 1-h test session under extinction conditions, following heroin self-administration training and 14 days of abstinence under FDR or sated conditions. Active lever responding was statistically significantly greater for the FDR group versus sated controls. * $p < .001$.

Heroin-trained-no-test group

Training. Mauchly's test of sphericity assumptions were violated for all training data. All values recorded were corrected using the Greenhouse-Geisser correction. The number of infusions increased over training days, and repeated measures ANOVA revealed a statistical trend, F (9,63) = 3.36, p = .075. Active lever responding did not result in an overall increase across training sessions, F (9,63) = 1.60, p = .245 as there was a peak in responding half way through the training period, which obscured a progressive increase over time. No statistically significant change in inactive lever responding was observed. Since some rats were removed from the analysis after the group matching was conducted (due to inadequate brain perfusion), last five days of training data was used to compare the rats assigned to the FDR (n=3) or Sated (n=5) groups. No statistically significant differences were found across all criteria; infusions, active lever responding and body weights.

Sacrifice day. On abstinence day 14, average body weights of the Sated group (M = 448.40, SEM = 26.43) were statistically significantly greater than the FDR groups (M = 347.33, SEM = 9.29) body weights, t (6) = -6.23, p = .001.

Drug-naive group

Following an 11 day period of unrestricted access to food in the ACF care facility, rats were separated into two groups, FDR (n=5) or Sated (n=5), matched according to mean body weights of the last five days. On day 25 in the ACF, average body weights of

the Sated group (M =472.00, SEM = 52.39) were statistically significantly greater than the FDR groups (M = 369.60, SEM = 28.06) body weights, t (8) = -3.85, p = .005.

Immunohistochemical Analysis

Nucleus accumbens shell. A statistically significant decrease in the number of Fos protein IR-labeled cells was observed for the FDR group, versus the Sated group, in the heroin-trained-tested rats, t (23) = -2.65, p = .014. No statistically significant differences were observed in the heroin-trained-no-test or drug-naive groups (see Figure 17).

Nucleus accumbens core. No statistically significant differences in Fos protein IR-labeling were observed in the heroin-trained-tested, heroin-trained-no-test or drug-naive groups (see Figure 18).

Basolateral amygdala. No statistically significant differences in Fos protein IR-labeling were observed in the heroin-trained-tested, heroin-trained-no-test or drug-naive groups (see Figure 19).

Central amygdala. No statistically significant differences in Fos protein IR-labeling were observed in the heroin-trained-tested, heroin-trained-no-test or drug-naive groups (see Figure 20).

Prelimbic cortex. No statistically significant differences in Fos protein IR-labeling were observed in the heroin-trained-tested, heroin-trained-no-test or drug-naive groups (see Figure 21).

Infralimbic cortex. No statistically significant differences in Fos protein IR-labeling were observed in the heroin-trained-tested, heroin-trained-no-test or drug-naive groups (see Figure 22).

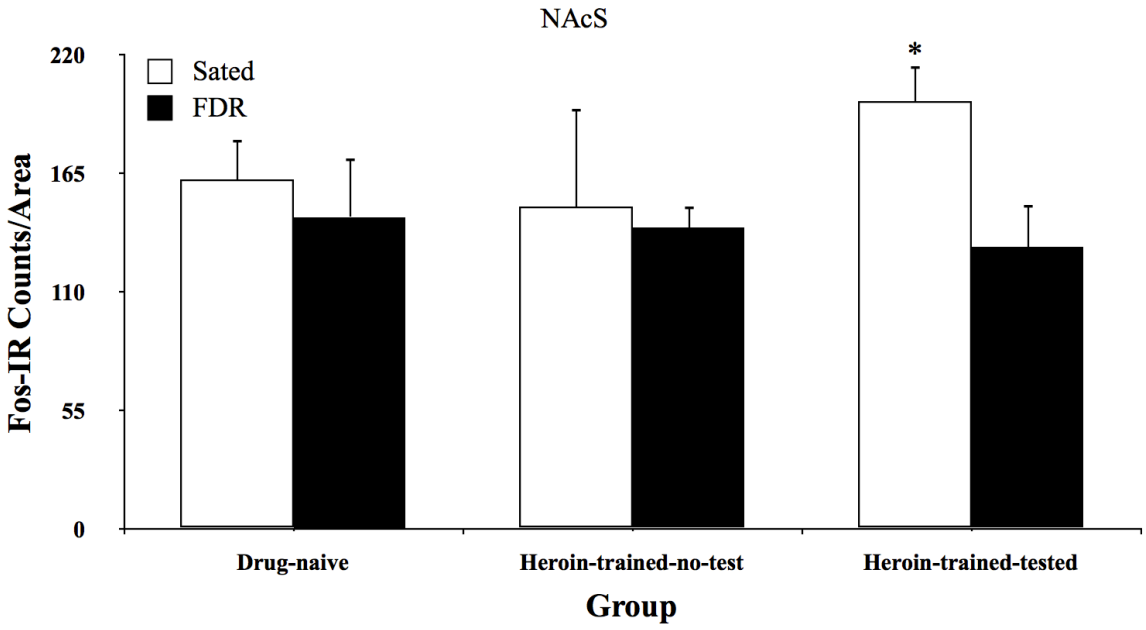

Figure 17. Fos protein IR-labeling in the nucleus accumbens shell. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred. *$p < .014$.

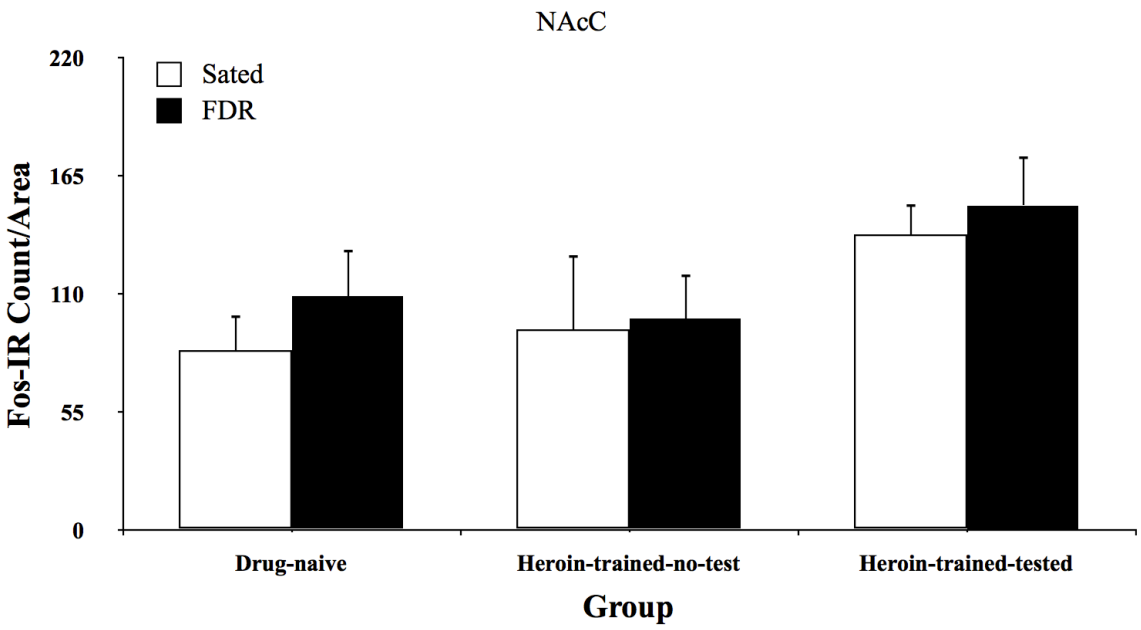

Figure 18. Fos protein IR-labeling in the nucleus accumbens core. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred.

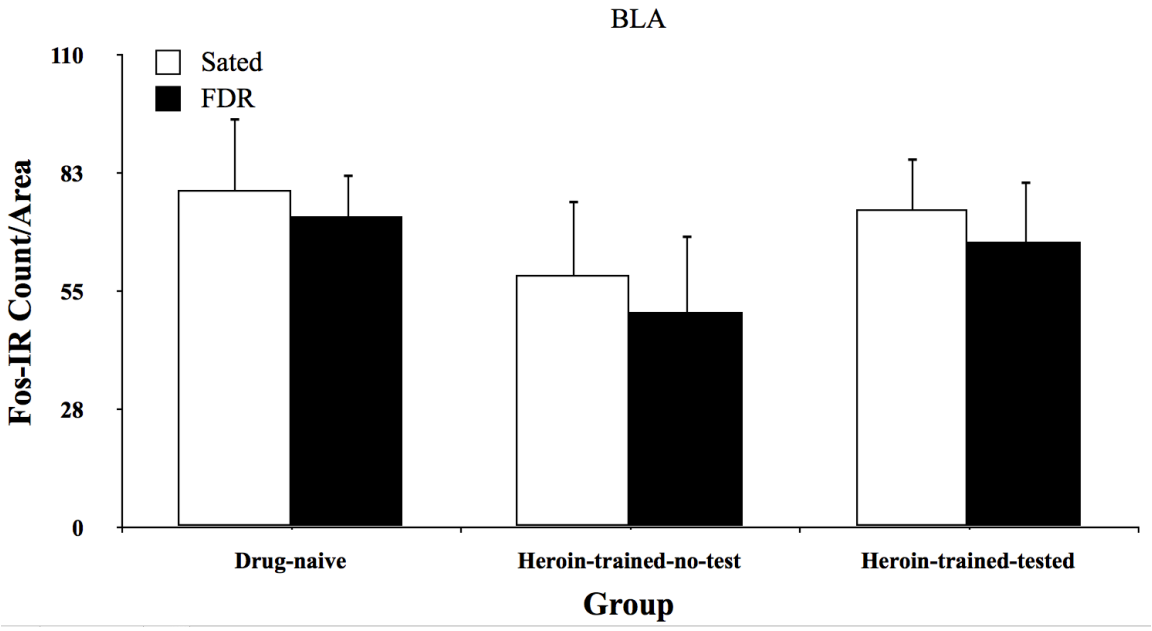

Figure 19. Fos protein IR-labeling in the basolateral amygdala. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred.

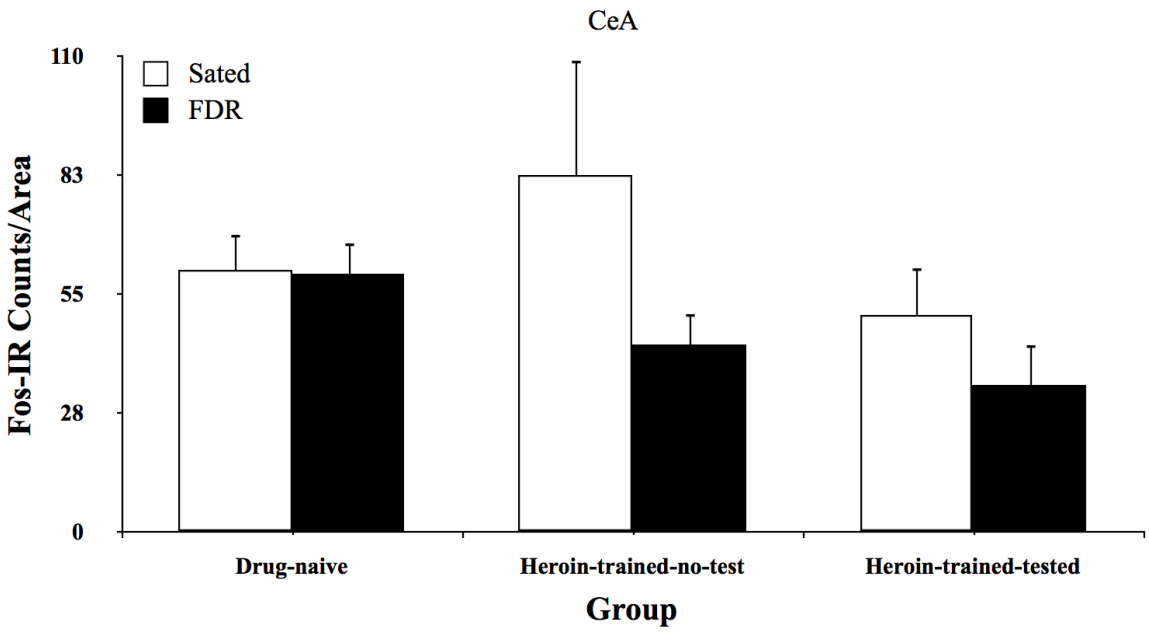

Figure 20. Fos protein IR-labeling in the central amygdala. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred.

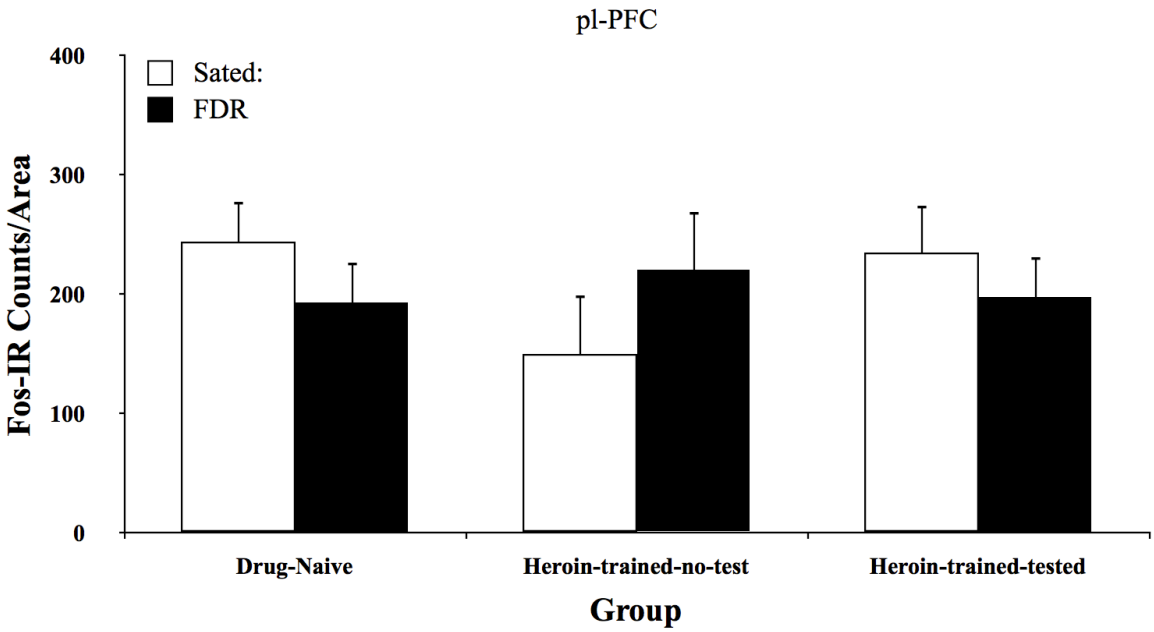

Figure 21. Fos protein IR-labeling in the prelimbic cortex. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred.

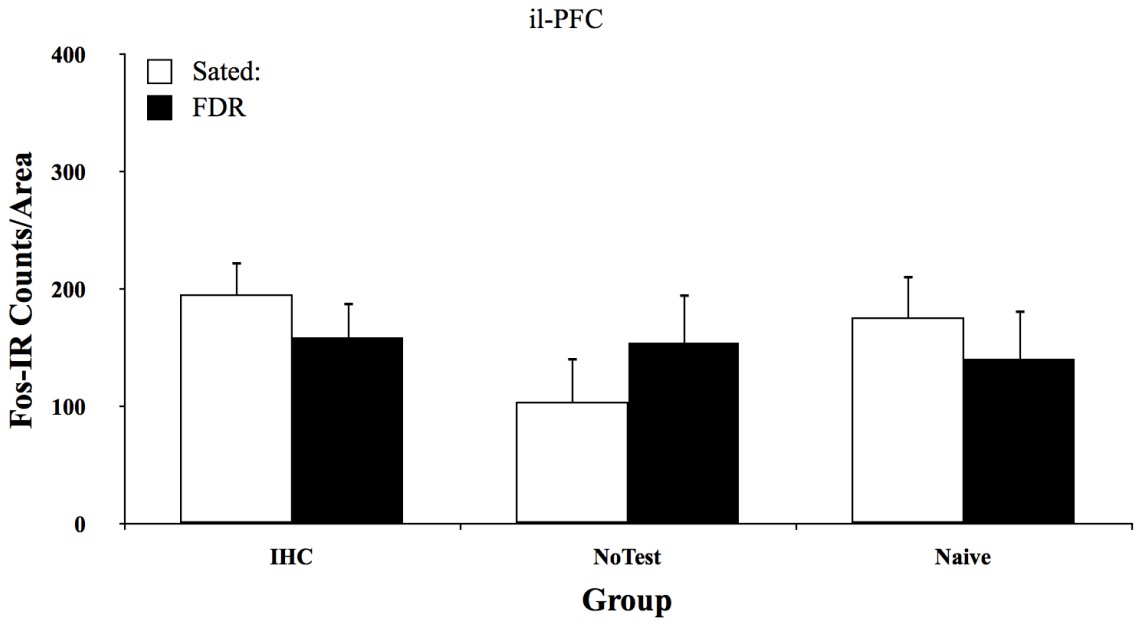

Figure 22. Fos protein IR-labeling in the infralimbic cortex. Data shown are the mean (+SEM) counts of Fos protein IR cells in the food restricted (FDR) and Sated group, for the heroin-trained-tested, heroin-trained-no-test and drug-naive groups. On abstinence day 14, rats were immediately sacrificed following a 1 h test under extinction conditions, or at the same time a test would have occurred.

Summary

As expected, a robust increase in heroin seeking was observed in food restricted, but not sated rats, when they were returned to the drug associated environment. Analysis of neuronal activation, as reflected by the expression of Fos protein, revealed a significant inhibition of Fos expression in the nucleus accumbens shell of the FDR compared to the sated rats in the heroin-trained-tested groups.

General Discussion

Recent work in our laboratory has demonstrated an augmentation of heroin seeking in abstinent, chronically food restricted rats (D'Cunha et al., 2012). Thus, as expected, a prolonged period of food restriction resulted in a robust increase in heroin seeking, compared to sated rats, across all experimental groups in the current studies. These findings are consistent with considerable evidence supporting a modulatory role for food restriction on drug-related behaviors in humans (Cheskin et al., 2005; Hall et al., 1992; Krahn et al., 1992) and in laboratory animals, where food deficiency drastically influences drug taking and the reinforcing properties of abused drugs (Carr, 2007; Carroll & Meisch, 1984; Stuber, Evans, Higgins, Pu, & Figlewicz, 2002).

The goal of the experiments described in Chapter 1 was to investigate the role of the CRF and corticosterone stress systems in the augmentation of heroin seeking by prolonged food restriction in abstinent rats. Treatment with R121919, a selective CRF_1-R antagonist, or α-helical CRF, a non-specific CRF-R antagonist, did not result in a statistically significant reduction in heroin seeking behavior. However, treatment with RU486, a glucocorticoid receptor antagonist, did not have an effect on heroin seeking. We therefore suggest that the acute stress response is not a critical factor in the augmentation of heroin seeking induced by prolonged food restriction. The experiments in Chapter 2 were therefore exploratory in nature and aimed at the identification of brain sites involved in this effect.

To that end, Fos protein immunoreactivity (Fos-IR) was used to measure neuronal activation in the BLA, CeA, NAcS and NAcC, il-PFC and pl-PFC. Interestingly, Fos-IR was statistically significantly attenuated in the NAcS of food restricted rats, compared to

the sated rats in the heroin-trained-tested group, while no other statistically significant effects were observed.

The role of stress systems in chronic food-restriction-induced augmentation of heroin seeking in the rat

Despite previous evidence demonstrating that CRF-R antagonists attenuate food deprivation-induced reinstatement of extinguished heroin seeking (Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003a), the experiments described in Chapter 1 suggest that these findings do not extend to chronic food restriction-induced augmentation of heroin seeking in abstinent rats. The observed lack of effect for CRF-R antagonists in chronically food restricted rats may be due to the use of different dietary regimens. As mentioned in the general introduction, chronic food restriction and acute food deprivation may differently affect metabolic systems and behavior. An additional reason for the different findings with acute and chronic food restriction might be the differences between the reinstatement procedure and our revised procedure. First, rats in the present study did not undergo a period of extinction, and as previously mentioned, extinction and abstinence can activate distinct neural circuits (Fuchs, Lasseter, Ramirez, & Xie, 2008a). Second, our study employed a prolonged period of mild stress (food restriction). Alterations in gene expression suggest distinct neural circuitry underly acute and chronic stress. For example, increased CRF_1-R and *c-fos* mRNA in the PVN of the hypothalamus are observed following acute, but not chronic stress. In contrast, chronic stress results in lowered levels of CRF_1-R and *c-fos* mRNA (Bonaz & Rivest, 1998). However, other reports have demonstrated the opposite result, where increased levels of CRF_1-R mRNA

were reported following chronic but not acute stress (Imaki, Nahan, Rivier, Sawchenko, & Vale, 1991). Notwithstanding these inconsistencies, there appear to be distinct adaptations in the CRF system following exposure to acute or chronic stress.

Additionally, it has been demonstrated that CRF can influence the long-term enhancement of synaptic transmission (long term potentiation, LTP) in the dentate gyrus of the hippocampus. For example, acute infusions of CRF in the hippocampus can result in long-lasting adaptations in the synaptic efficacy of hippocampal neurons, an effect which is abolished by CRF-R antagonist pre-treatment (Wang, Wayner, Chai, & Lee, 1998). The hippocampus projects to the NAc and is strongly implicated in memory formation and the contribution of emotional memories to addiction (Nestler, 2005a). Chronic exposure to cocaine, opiates and nicotine has been shown to inhibit the birth of new neurons in the hippocampus (Eisch, 2000; 2002). Moreover, chronic exposure to cocaine can also stimulate dendritic growth in the NAc, which consequently allows for an enhanced influence on NAc neurons by afferent projections from surrounding regions such as the hippocampus (Nestler, 2005a). Nestler and colleagues (2001; 2005b) have suggested that cognitive impairments in drug addicted individuals may be driven by these long-lasting alterations in hippocampal neurons, and in turn in their influence on the reward circuitry. Therefore, the connecting circuitry between the hippocampus and other brain regions within the mesocorticolimbic DA circuitry suggests a pathway by which CRF can act to cause long lasting adaptations that influence drug seeking behavior.

In the present study, rats were exposed to a 14 day food restriction stress, which may have resulted in adaptations in critical neuronal circuits long before the test session. For example, greater DA tissue levels in the NAc, and reduced levels in the PFC, were

found 1 week following a 13 day exposure to CRF (Izzo, Sanna, & Koob, 2005). Thus, acute CRF-R antagonist treatment prior to testing may not have an important influence on the CRF-induced adaptations in the current procedure. Future studies should investigate the effects of chronic CRF-R antagonist treatment, over the food restriction period, in the augmentation of heroin seeking induced by chronic food restriction. A differential role for acute versus chronic treatment with CRF antagonists is suggested by the findings of Mallo and colleagues (2004) who reported a reduction in anxiety (as defined by increased exploration) in an elevated-zero-plus-maze test following chronic, but not acute treatment with a selective CRF_1-R antagonist.

Null effects in the present study (Chapter 1; Experiment 1) may have been due to the choice of CRF-R antagonists. R121919 and α-helical CRF were used, the former a selective CRF_1-R antagonist and the latter a non-selective CRF-R antagonist, with a high affinity for CRF_1 and CRF_2-Rs (Behan et al., 1996). As mentioned in the introduction of Chapter 1, CRF_2-Rs may be involved in increased anxiety-like behaviors and drug self-administration (Funk & Koob, 2007) however, their role in stress is not clear (Bale & Vale, 2004). For example, ethanol dependent rodents decrease ethanol self-administration in response to intra-CeA infusions of urocortin 3 (Ucn_3), a highly selective CRF_2-R *agonist* (Funk et al., 2007). In contrast, intra-CeA infusion of Ucn_3 increased ethanol, but not water, self-administration in non-ethanol dependent rats (Funk & Koob, 2007). The authors further suggest that CRF_1-R and CRF_2-R may have opposing actions in the basal forebrain. Wang and colleagues (2007) have also demonstrated that CRF_2, but not CRF_1-R blockade in the VTA can reduce elevated glutamate and DA concentrations and attenuate footshock stress-induced reinstatement of cocaine seeking (Wang et al., 2005).

Taken together, these studies suggest that CRF_2-Rs activation at particular brain areas might have a role in drug seeking, and therefore the results in the present study should be interpreted with caution as the CRF_2-Rs were not specifically manipulated, and no site-specific injections were used. Nevertheless, the fact that blockade of both CRF_1-R and CRF_2-Rs using a non-specific antagonist did not affect drug seeking strongly suggests that acute activation of the CRF system is not involved in this phenomenon.

An interesting, albeit not statistically significant, trend for a dose dependent reduction in responding on the previously heroin paired (active) lever on the test day was observed in the α-helical CRF-treated sated group. Recently, CRF-R antagonism was shown to reduce cue-induced reinstatement of drug seeking (Moffett & Goeders, 2006), which could provide a possible explanation for the reduction of active lever responding observed in sated rats in the current experiments, following exposure to the drug-associated environment and cues. However, a similar pattern was found for inactive lever responding in the food restricted and sated groups, suggesting that the reduced lever seeking in the α-helical CRF-treated rats was not due to changes in the motivational value of the drug-associated stimuli. Furthermore, administration of R121919 did not reduce active or inactive lever responding in the drug treated groups, further supporting a lack of motivational effects for CRF-R antagonists in the current procedure. It is possible that treatment with α-helical CRF resulted in an overall reduction of locomotor responding, which was obscured by the increased drug-seeking behavior in the food restricted rats; yet, we found no indication for such an effect in the previous studies conducted in our laboratory (Shalev et al., 2006).

Since the treatment with α-helical CRF had no effect on drug seeking in the food restricted group, the administration of α-helical CRF was investigated under anxiety provoking conditions to ensure the efficacy of the drug. In this test, a reduction in anxiety, as assessed by a reduction in the latency to consume food and the number of approaches prior to food consumption, in food restricted rats that received α-helical CRF treatment was found. Rats that did not receive the drug treatment approached the food multiple times with no attempt at consumption and would instead continue to explore the environment. We interpreted this to be a sign of conflicting behavior resulting from elevated anxiety, as the rats were clearly hungry.

Given the apparent absence of a role for CRF (Chapter 1; Experiment 1) in protracted food restriction-induced augmentation of drug seeking, we investigated the role of corticosterone, the major stress-associated hormone (Chapter 1; Experiment 2). ACTH and the subsequent production of corticosterone can also be affected by mechanisms independent of CRF's actions in the HPA axis (Tsigos & Chrousos, 2002). Previous research suggests an increase in plasma corticosterone concentrations as a result of reward and stress (including dietary restriction) presentations (Burgess et al., 1993; Goeders, 1997; Heiderstadt, McLaughlin, Wright, Walker, & Gomez-Sanchez, 2000; Merali, McIntosh, Kent, Michaud, & Anisman, 1998; Szechtman, Lambrou, Caggiula, & Redgate, 1974). These stress-induced elevations in corticosterone, however, are thought to have no role in stress-induced reinstatement of drug seeking (Erb et al., 1998; Shaham et al., 1997; Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003a). It is relevant to note however, that the majority of these reports manipulated drug taking with acute stressors, and while stressors such as acute food deprivation can elevate plasma concentrations of

corticosterone, they do so at lower levels compared with other stressors such as cold or heat (Djordjević, Cvijić, & Davidović, 2003). Chronically food restricted rodents exhibit greater levels of corticosterone compared to controls (Carr, 1996). More so, the elevated concentrations observed following food restriction are positively associated with the proclivity to self-administer cocaine. Additionally, the removal of corticosterone via aderenalectomy can also decrease the heightened locomotor activity to a psychostimulant challenge in food restricted rats (Deroche et al., 1995; Piazza & Le Moal, 1996).

Thus, it was crucial to follow up Experiment 1 by investigating the role of corticosterone in our revised model. As we expected, acute treatment with a glucocorticoid antagonist, RU486, did not reduce increased heroin seeking in rats with a history of chronic food restriction. These results are consistent with past studies in the literature on stress- and reward-related behaviors. First, in CRF deficient mice, activity in an anxiety provoking situation (e.g., elevated plus maze) remains unaffected, in spite of a sufficiently blunted HPA axis response and lowered concentrations of corticosterone (Dunn & Swiergiel, 1999). Therefore, a heightened physical stress response may not always be necessary for the expression of stress-related behaviors. Second, Abrahamsen and colleagues (1996) suggested that in an LHSS procedure, the sensitization of rewarding efficacy of the stimulation by food restriction is unaltered following a treatment with a corticosterone synthesis inhibitor, or a feeding-induced decreases in plasma corticosterone (Abrahamsen, Berman, & Carr, 1995). The aforementioned studies argue against a modulatory role for corticosterone in rewarding behaviors. As Carr (2002) suggests, however, the most comprehensive test of corticosterone's involvement in food restriction would be to maintain corticosterone concentrations in the food

restricted group at similar concentrations as those reported in the sated controls over the full period of restriction. With this recommendation in mind, we suggest that future studies investigate the long-term elevation of corticosterone by food restriction and whether this elevation may lead to adaptations that cannot be manipulated by the acute inhibition of corticosterone.

To ensure that RU486 successfully blocked corticosterone binding to its receptors, tail blood was collected immediately after the test session in the operant conditioning chambers. As expected, concentrations of corticosterone were statistically significantly greater in food restricted and sated rats after treatment with RU486 compared to the vehicle pretreatment. Interestingly, the magnitude of increase in the food restricted group (~ 500%) was greater than that in the sated group (~ 74%) following RU486 treatment. We speculate that these differences in magnitude can be explained by a food restriction-induced elevation of corticosterone. One point of interest is that RU486 is not selective to glucocorticoid receptors, but shows progesterone receptor antagonist properties as well. In fact, it possesses greater relative binding affinities (RBA) for progesterone (RBA: 530) compared to glucocorticoid (RBA: 300) receptors (Moguilewsky, 1985); this lack of receptor specificity however, is not relevant here as null effects were observed following administration of RU486 in our procedure.

Another interesting pattern in the current study (Chapter 1; Experiment 2) involves a non-statistically significant pattern of decreased active and inactive lever responses following RU486 treatment. This is in agreement with findings from previous studies that reported an attenuation of locomotor responding in cocaine treated rats after RU486 injections (Wu et al., 2008). These effects however, were observed using 3 mg/kg

and not in a 25 mg/kg dose (Wu et al., 2008), the latter of which is closer to the 30 mg/kg used in the present study. Additionally, a dose of 25 mg/kg of RU486 did not reduce wheel running after a fasting-induced increase in locomotor activity was observed (Challet, Lemaho, Robin, Malan, & Cherel, 1995). Thus, the literature has not provided any consistent reports on the effects of RU486 on locomotor activity. Future studies should investigate the removal of adrenal glands by means of adrenalectomy in combination with corticosterone replacement to elucidate the role of corticosterone or progesterone specifically. Removal of the adrenal glands is particularly important as it will avoid the receptor antagonist-induced augmentation of corticosterone levels observed in the present study, which may have unexpected effects on drug seeking behavior. This last point may be pertinent as the removal of adrenal glands has been effective in blocking stress-induced potentiation of conditioned place preference to morphine (Der-Avakian et al., 2005).

In conclusion, we suggest that the acute stress response has no role in the augmentation of heroin seeking by prolonged food restriction. There is evidence that the augmentation of drug seeking following food restriction can be modulated by non-stress related mechanisms that are triggered by the hunger state (Cabeza de Vaca & Carr, 1998), which provide alternate mechanisms of action that drive heroin seeking in our procedure. In agreement with this suggestion, recent evidence from our laboratory has reported that a 2 or 24 h re-feeding period eliminated the augmentation of heroin seeking induced by food restriction after a period of abstinence (D'Cunha et al., 2012). The hunger state alone is not sufficient to drive drug seeking in our procedure, however, and requires a history of prolonged food restriction as well. Similar dietary manipulations have been

shown to alter hormonal and homeostatic mechanisms. For example, reductions in plasma and brain insulin concentrations represent an alternative mechanism by which chronic food restriction can regulate reward-related behaviors (Cabeza de Vaca & Carr, 1998; Woods et al., 1985). There is evidence that chronically decreased plasma concentrations of insulin, in diabetic animals, results in reductions in DA transporter (DAT) mRNA in the VTA and substantia nigra (SN) (Figlewicz, Brot, McCall, & Szot, 1996). In contrast, central injections of insulin increase expression of the DAT mRNA in the VTA and SN (Figlewicz, Szot, Chavez, Woods, & Veith, 1994). This suggests the involvement of insulin in the sensitization of the brains reward circuitry.

As well, two additional hormones, leptin and ghrelin, which are involved in energy balance and body weight regulation, have been linked to the modulation of drug and natural rewards through their effects on the mesocorticolimbic DA circuitry (Cummings, Naleid, & Figlewicz Lattemann, 2007). Infusions of ghrelin, an orexigenic gut hormone, can increase extracellular DA concentrations in the NAc (Jerlhag et al., 2007), and cocaine-induced increases of extracellular DA in the NAc are attenuated by ghrelin receptor antagonism (Jerlhag, Egecioglu, Dickson, & Engel, 2010). In ghrelin knockout mice, alcohol-induced increases in NAc DA levels is suppressed (Jerlhag, Landgren, Egecioglu, Dickson, & Engel, 2011), while treatment with ghrelin receptor antagonists in alcohol dependent rats was shown to attenuate self-administration of ethanol (Landgren et al., 2011). Importantly, increased serum concentrations of ghrelin have been observed in response to cue-induced reinstatement of cocaine seeking (Tessari et al., 2007). Treatment with a ghrelin antagonist however, did not impair the food deprivation-induced reinstatement of heroin seeking, although central infusions of ghrelin

did increase the breakpoints on a progressive ratio schedule of heroin reinforcement (Maric, Sedki, Ronfard, Chafetz, & Shalev, 2011). It is critical to note, however, that the aforementioned study used acute food deprivation in a reinstatement of extinguished drug seeking procedure, which as mentioned above might involve different brain mechanisms than prolonged food restriction in abstinent rats.

Leptin, an anorexigenic hormone that is secreted by peripheral adipocytes, can regulate activity in the mesocorticolimbic circuitry (Cummings et al., 2007) through its actions on VTA DA neurons, and has been implicated in reward processes. Interestingly, leptin was shown to attenuate acute food deprivation-induced reinstatement of heroin seeking (Shalev, Yap, & Shaham, 2001b). However, this effect was not consistent across stress- (footshock) or heroin priming-induced reinstatement (Shalev et al., 2001b), suggesting that leptin's effect was not mediated by DA or stress-related pathways.

The hormones described above may present alternative mechanisms by which the augmentation of heroin seeking following prolonged food restriction is achieved.

The effects of chronic food restriction on Fos immunoreactivity in the nucleus accumbens in heroin seeking rats

In Chapter 2 we described a reduction of Fos protein-immunoreactivity (Fos-IR) in the NAcS for heroin-trained-tested rats who where subjected to a prolonged food restriction, compared to those with unrestricted access to food (sated). This is in contrast to the report that heightened Fos-IR levels are observed in the NAcS after repeated stress exposure (Nikulina, Covington, Ganschow, Hammer, & Miczek, 2004), and that transient inhibition of the NAcS using GABA agonists attenuates a footshock stress-induced

reinstatement of cocaine seeking (McFarland et al., 2004). Carr and colleagues (2000), however, reported increases in Fos-IR only in amphetamine challenged, but not saline control rats using a more severe regiment of food restriction.

The food restriction-induced inhibition of neuronal activation in the NAcS we report here is in agreement with recent findings indicating that the NAcS may play a bidirectional role in drug seeking, depending on DA and glutamatergic inputs. Thus, AMPA receptor (a glutamate receptor) activation in the il-PFC (which results in glutamate release in the NAcS) resulted in a suppressed cue-induced reinstatement of cocaine seeking. This effect was reversed by intra-NAcS infusions of DA, activation of VTA DA neurons (thereby increasing DA release in the NAc), or by AMPA receptor antagonists (LaLumiere, Smith, & Kalivas, 2012a). These findings suggest that the existence of high extracellular DA concentrations or impaired glutamatergic input to the NAcS is necessary for the expression of drug seeking. The authors further speculate that DA release in the NAcS might *inhibit* neuronal activation, resulting in the enhanced expression of drug seeking. It should be noted, however, that the aforementioned studies investigated drug seeking following psychostimulant, but not opiate drug use. Nevertheless, preliminary findings in our laboratory show an increase in extracellular DA in food restricted, abstinent rats with a history of heroin self-administration, during a test for drug seeking (D'Cunha, Hamel, Sedki, & Shalev, 2012).

Thus, in the present study, the reduction of Fos-IR in the NAcS of food restricted compared to sated rats in the heroin-trained-tested group may be the result of increased DA release following exposure to the drug-associated stimuli, resulting in the augmentation of drug seeking. A similar reduction in Fos-IR in the food restricted group

was not observed in the drug-naive or heroin-trained-no-test rats (that were not exposed to the drug environment).

Future studies should investigate this reduction in Fos-IR following non-contingent exposure to heroin, and sucrose or saline self-administration. First, these studies can examine whether self-administration as compared to passive exposure to the drug is necessary for the decrease in NAcS Fos-IR. Second, they can clarify whether reduced activation in the NAcS is associated with exposure to drug-conditioned stimuli as compared to natural rewards-conditioned stimuli. Additionally, in the present procedure rats were exposed to contextual and discrete cues during the test session. As mentioned in Chapter 2, the NAcS is critical for context induced-drug seeking. Further studies should examine the particular contribution of the discrete and contextual cues. For example, the drug context could be extinguished during the prolonged food restriction prior to the test session, and Fos-IR analyzed as in the current study.

Previous reports have demonstrated elevated Fos-IR expression in the il-PFC following the context-induced reinstatement of heroin seeking. Furthermore, a reduction in context-induced reinstatement of heroin seeking is observed following transient inhibition of the il-PFC by GABA agonists (Bossert et al., 2011). Furthermore, an increase in Fos-IR was observed in the il-PFC cortex following a test for the renewal of extinguished cocaine seeking (Hamlin, Clemens, & McNally, 2008). We therefore expected a rise in Fos-IR in the il-PFC in food restricted rats compared to sated controls; however no increase in activation was observed. It is worth noting that in the above-mentioned studies, drug seeking was extinguished prior to reinstatement tests. It is possible that the il-PFC is involved in the extinction of conditioned drug seeking and not

necessarily drug seeking itself. In the present study rats underwent a period of abstinence, but not extinction, which could explain the absence of changes in Fos-IR.

The absence of any distinct activation in the NAcC or pl-PFC between the food restricted and sated groups was unexpected, particularly because the pathway from the prelimbic cortex (pl-PFC) to the NAcC has been strongly implicated in the potentiation of drug-seeking behavior (LaLumiere & Kalivas, 2008; Peters, Kalivas, & Quirk, 2009). Increased output of extracellular glutamate is observed in the pathways from the pl-PFC to the NAcC during a cocaine-primed reinstatement (Baker et al., 2003). Moreover, increased extra-cellular levels of glutamate have been measured in the NAcC during footshock stress-induced reinstatement of cocaine seeking, and this increase was blocked by dorsal PFC inactivation (McFarland et al., 2004). We speculate that heightened neuronal activation in the NAcC may have occurred in the heroin-trained-tested, but no differences were observed as the food manipulation did not affect such activation, suggesting that the augmentation of drug seeking in the food restricted rats is not related to neuronal activation in the dorsal PFC. This idea is supported by the fact that no differences in Fos-IR between the drug-naive food restricted and sated groups were observed. Perhaps a comparison between the food restricted heroin-trained-tested group versus the food restricted heroin-trained-no-test or drug naive rats would have revealed increased activation during the drug seeking test. The purpose of the current study, however, was to elucidate the differences between food restricted and sated rats and therefore not all possible comparisons were performed, in an attempt to increase the power of the statistical analysis.

The absence of differences in Fos-IR in the pl-PFC between the feeding conditions was particularly interesting, as acute food deprivation-induced reinstatement of heroin seeking resulted in an augmentation of Fos-IR in the pl-PFC (Shalev, Robarts, Shaham, & Morales, 2003b). However, rats in the aforementioned study were subjected to a period of extinction, and while the pl-PFC is integral to the modulation of drug seeking after extinction training, it is not necessary for the expression of drug seeking following a period of abstinence without extinction training (Fuchs et al., 2006). Furthermore, while there is evidence that supports greater Fos-IR levels in the pl-PFC after long-term abstinence from psychostimulant drugs (Ciccocioppo, Sanna, & Weiss, 2001), there is no literature on the effects of prolonged food restriction.

As previously suggested in the introduction to Chapter 2, the amygdala is implicated in stress, drug reward, conditioned drug rewards and relapse. For example, lesions of the BLA, but not the NAc attenuated responding for a cocaine-paired conditioned stimulus (Grimm & See, 2000; Whitelaw et al., 1996). Moreover, stimulation of the BLA results in the reinstatement of cocaine and amphetamine seeking (Hayes et al., 2003; Taepavarapruk & Phillips, 2003; Vorel, Liu, Hayes, Spector, & Gardner, 2001). However, Fuchs and colleagues (2006; 2005) have demonstrated that the BLA is critical to the expression of the context-induced reinstatement of extinguished cocaine seeking but not to the augmentation of drug seeking following protracted abstinence.

Lastly, although exposure to a drug discriminative stimulus following abstinence from cocaine has been reported to increase Fos-IR in the BLA (Ciccocioppo et al., 2001), drug-cue-induced increases in neuronal activation would have been reflected in both the food restricted and sated groups, and apparently it is not affected by feeding condition.

This conclusion is supported by the lack of differences in Fos-IR between FDR and sated rats in the drug-naive and heroin-trained-no-test-groups.

No effects in Fos-IR where observed in CeA. This region was chosen as it is a critical component of the brains stress circuit (Kalivas & McFarland, 2003), and is involved in expression of drug seeking behavior in response to stress (Kalivas & Volkow, 2005). Our data, however, are consistent with previous findings indicating no distinct patterns of Fos-IR in the CeA in an acute food deprivation-induced reinstatement of heroin seeking when compared to sated controls (Shalev, Robarts, Shaham, & Morales, 2003b). Finally, since the findings described in Chapter 1 suggest that the stress response is not involved in food restriction-induced augmentation of drug seeking in our procedure, the lack of differences in CeA activation is not an unexpected result.

One limitation of the Chapter 2 experimental procedure is the difficulties in the use of Fos-IR labeling, which may result in inconsistencies. For example, *c-Fos* has a generally low activation threshold in certain brain areas and therefore neuronal deactivation (inhibition) is difficult to identify. A second limitation was the low number of rats in the heroin-trained-no-test group (*n's*: FDR - 3, Sated - 5). These small sample sizes resulted in a large degree of variability within each group and a reduced power. Also, the method in which animals were prepared for perfusion may have led to changes in Fos protein expression. As previously mentioned, Euthenol was used to anesthetize the rats prior to perfusion. In some cases the animal took almost 40 minutes to be fully anesthetized. This time was undoubtedly very stressful for the rat and may have inadvertently led to increased Fos-IR expression in some regions. Finally, time of sacrifice following testing used here might not have been optimal. Rats were sacrificed

immediately following a 60 min test session. Others have often used a 90-120 min session or a 60 min session followed by a 30 min time out prior to the sacrifice (D'Este, Scontrini, Casini, Pontieri, & Renda, 2002; Darcel et al., 2005; Dayas, Liu, Simms, & Weiss, 2007; Shalev, Robarts, Shaham, & Morales, 2003b). It is therefore possible that we missed the peak of the Fos activation, resulting in less than ideal conditions to identify subtle differences between groups.

Analysis of all the brain regions involved in drug reward, stress and drug seeking is beyond the scope of this thesis and therefore we briefly mention other brain sites that may provide insight on the mechanisms that underlie the augmentation of heroin seeking in our procedure. These brain sites present viable targets for future analysis due to their critical role in the expression of conditioned drug rewards following abstinence. Kalivas and colleagues (2005) have indicated that the ventral palladium is crucial as it contributes to a "final common pathway" which drives drug-seeking behavior. The same group has also proposed a role for the substantia nigra, dorsal striatum and somatosensory cortex in the expression of drug seeking following abstinence (Kalivas, 2008). Furthermore, the orbitofrontal cortex (OFC) is also implicated in conditioned drug rewards following protracted abstinence, as increased Fos-IR levels are found in this region following tests for cocaine seeking in abstinent rats (Zavala, Biswas, Harlan, & Neisewander, 2007). Finally, studies have suggested that elevated Fos-IR levels in the VTA are observed during a cue-induced test for cocaine seeking, following prolonged abstinence (Kufahl et al., 2009). Together, these brain sites present viable targets for future analysis due to their critical role in the expression of conditioned drug rewards following abstinence.

Conclusion

In conclusion, we present here a set of experiments (Chapter 1) which indicate that pathways involved in the acute response to stress are not critical for the expression of augmented drug seeking in abstinent, food restricted rats. The long-term manipulation of CRF and the corticosterone stress systems, from the inception of the food restriction period to the test session, may elucidate the role of the these hormones in our procedure.

Data from Chapter 2 demonstrated that food restriction in heroin-trained-tested rats decreased Fos-IR levels in the NAcS, when compared to sated controls. The finding that food restriction selectively decreased Fos-IR in the heroin-trained-tested but not the heroin-trained-no-test or drug-naive groups suggests that this effect is specific to drug seeking and not a general response to food restriction. Finally, our finding is in agreement with a recently suggested model that postulates that drug seeking is driven by an inhibition of neuronal activation in the NAcS that can result from enhanced DA release following exposure to the drug, drug-associated stimuli, or stress (LaLumiere, Smith, & Kalivas, 2012b).

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Appendix A: Project Timeline

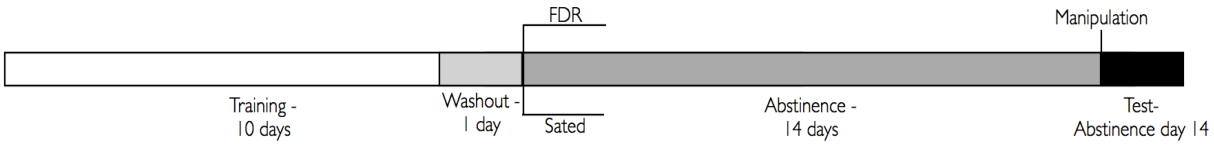

Figure A1. Timeline of experimental procedure. The procedure consists of three phases: animals are first trained to self-administer a drug in the presence of a cue/tone complex (training phase), then moved to a different context and undergo a one day, drug washout period, followed by a prolonged period of food restriction (FDR) or unlimited access to food (abstinence phase) and finally returned to the self-administration environment for a drug-seeking test in the presence of drug-paired cues under extinction conditions (test phase).