Augmentation of heroin seeking following chronic food restriction in the rat: A role for nucleus accumbens dopamine

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ABSTRACT

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Previous research using an animal model of relapse has shown that acute food deprivation will increase drug seeking. Recent evidence from humans, however, suggests that chronic food restriction rather than acute deprivation is related to increases in drug taking and relapse, emphasizing the need to develop an animal model to elucidate the mechanisms mediating the effects of chronic food restriction on drug seeking. We studied the effects of chronic food restriction during a period of abstinence on heroin seeking in rats. Results demonstrated an augmentation of heroin seeking in chronically food restricted rats with a history of heroin self-administration. Re-feeding prior to the drug seeking test or decreasing the length of the food restriction period prevented the augmentation of drug seeking. A combination of chronic food restriction and a concurrent state of hunger appear to be necessary for the augmentation of heroin seeking induced by food restriction. Previously, it was demonstrated that chronically food restricted rats display alterations in the mesolimbic dopamine system, a critical component of the reward system. Consequently, we assessed extracellular levels of dopamine in the nucleus accumbens, one of the major targets for mesolimbic dopamine neurons, during the drug seeking test, following chronic food restriction in abstinent rats with a history of heroin self-administration. Preliminary data indicate significantly higher levels of dopamine throughout the drug seeking test in the food restricted rats. Our findings suggest that food restriction-induced changes in dopamine release in the nucleus

accumbens are associated with the augmentation of drug seeking in food restricted abstinent rats.

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General Introduction

Addiction is a chronic relapsing disorder characterized by compulsive drug seeking and a loss of control over drug consumption (O'Brien & McLellan, 1996). Although drug taking begins as a voluntary behavior, once addiction develops, strong urges to continue drug taking can overwhelm an individual (O'Brien, 1997). Consequently, despite efforts to refrain from drug use following detoxification programs and periods of abstinence, relapse frequently occurs (O'Brien, 1997). The chronic reoccurring nature of the disorder makes a major contribution to the heavy price paid by addicts and society. The burden placed on the health care, criminal justice, and other social systems by the approximately 200,000 Canadians dependent on an illicit drug accumulating costs of over eight billion dollars annually (Tjepkema, 2004). Heroin users, in particular, exhibit a life characterized by repeated cycles of drug abuse and abstinence, along with an increased risk of crime, health problems, and premature death. In heroin users that were followed over three decades, 40% of the participants still reported heroin use at the end of the study. However, those that had achieved 5 years of abstinence showed promise of recovery. Despite this success, even after 15 years of abstinence, 25% of heroin dependent subjects in the study had relapsed, indicating that physical withdrawal symptoms were not a factor since acute withdrawal symptoms typically last 24-72 hours (Alper, Lotsof, Frenken, Luciano, & Bastiaans, 1999; Hser, Hoffman, Grella, & Anglin, 2001). Moreover, the death rate among heroin dependent subjects is estimated to be 50-100 times greater than that of the general population in the same age range (Hser, et al., 2001). These findings indicate that heroin addiction is a chronic and debilitating disorder characterized by a number of maladaptive and lethal consequences.

Understanding the underlying mechanisms of this disease will help develop better treatment programs that are effective and long-lasting.

In abstinent drug users, drug craving and relapse are triggered by three main factors. First, re-exposure to the previously abused substance, known as drug priming, has been shown to increase the subjective craving and desire for the drug, leading to drug relapse (de Wit, 1996). Second, cues that were previously paired with the availability and consumption of the drug, such as the proximal and distal cues relating to a drug, the drug-taking context and drug paraphernalia, may also elicit arousal and craving responses, leading to the resumption of drug taking (Childress et al., 1993). Lastly, stressors of various forms may also trigger relapse to drug use (Sinha, 2001). Clinical populations of drug users report that increased craving and drug relapse are often precipitated by stressful life events and situations (Hyman, Fox, Hong, Doebrick, & Sinha, 2007; Sinha, 2001). However, establishing a causal relationship between stress and drug relapse is difficult in clinical environments since the observations in these settings are correlational and may be biased because of retrospective recall of stressful events (Sinha, Shaham, & Heilig, 2011). However, laboratory studies have also established a clear causal link between stress exposure and drug craving (Sinha, et al., 2011). Using methods such as guided imagery of stressful events, with individualized scripts, it was reported that subjective self-reports of drug craving are increased (Sinha, 2001, 2009). In addition, prospective studies demonstrate that both acute and chronic stressors are associated with subsequent drug relapse (Brown, Vik, Patterson, Grant, & Schuckit, 1995; Preston & Epstein, 2011; Sinha, 2001).

A relatively common stressor in humans is restricted food intake. Indeed dietary restriction leads to reports of increased drug craving and a higher incidence of relapse across a variety of situations, an effect that has been documented by psychologists outside laboratory settings. For example, restricted food intake during times of war leads to increased intake of coffee and tobacco products (Franklin, Shiele, Brozek, & Keys, 1948). Restricted diet also leads to increased coca leaf chewing in malnourished Peruvian Indians (Hanna & Hornick, 1977). Furthermore, the severity of dieting in young women is positively associated with the prevalence of alcohol, cigarettes, and marijuana use (Krahn, Kurth, Demitrack, & Drewnowski, 1992), and empirical evidence demonstrates that food restriction increases the risk for relapse in abstinent smokers (Hall, Tunstall, Vila, & Duffy, 1992).

Importantly, unbalanced diet and insufficient nutrition is a common problem among heroin users. Active heroin users report eating infrequently, and display a loss of interest in food (Neale, Nettleton, Pickering, & Fischer, 2012). However, following treatment and cessation of heroin use, appetite returns along with weight gain. This increase in weight leads to increased anxiety levels about becoming overweight, and controlling appetite becomes a major concern for abstinent users (Neale, et al., 2012). Therefore, dietary restriction, or restricted food intake is a pressing issue of consideration in the development of addiction treatment programs, more so since a high comorbidity rate also exists between eating disorders and substance abuse disorders (Harrop & Marlatt, 2010; Holderness, Brooks-Gunn, & Warren, 1994).

In summary, stressors, such as dietary restriction, are related to increased drug craving and a higher risk for relapse. However, the precise mechanisms underlying these

effects remain unknown. Clinical studies provide only correlative data between stress and relapse (Sinha, et al., 2011), making it difficult to identify precise causal relationships. In addition, although laboratory studies in humans do demonstrate cause-effect relationship between stress and drug craving, craving, by itself does not always lead to relapse. Finally, ethical constraints limit the type of research that can be conducted on human subjects to elucidate the neural underpinnings of stress-induced relapse. Animal models can provide a useful approach to studying the neurobiology underlying stress-induced relapse, as they allow for a greater control of experimental parameters and may utilize approaches otherwise deemed unethical in human subjects (Shaham, Shalev, Lu, de Wit, & Stewart, 2003).

The reinstatement procedure is a widely adopted model of relapse and has been demonstrated to have both face and predictive validity (Epstein, Preston, Stewart, & Shaham, 2006; Shalev, Grimm, & Shaham, 2002). Reinstatement is defined as the resumption of a behavior that has been previously extinguished (Bouton & Swartzentruber, 1991). In the reinstatement procedure, animals are trained to self-administer a drug (e.g. heroin or cocaine). After stable self-administration is established, responding for drug infusions is extinguished by the removal of the drug. Once the extinction criterion has been reached, a trigger is used to elicit renewed drug seeking under extinction conditions. The advantage of investigating drug seeking under extinction conditions, as opposed to when drug is available, is that it avoids the direct psychomotor effects of the drug on behavior. Studies using the reinstatement model have demonstrated that the same factors that trigger drug craving and relapse in humans also reinstate drug

seeking in animals (de Wit & Stewart, 1981; Meil & See, 1996; Shaham & Stewart, 1995).

The reinstatement procedure and other animal models of drug consumption and relapse, indicate that dietary manipulations can affect drug intake and drug seeking in animals the same way that they affect humans. That is, both acute food deprivation and chronic food restriction (defined as prolonged exposure to limited access to, or to limited amount of food) increase self-administration of many types of drugs (Carroll, France, & Meisch, 1979; Carroll & Meisch, 1981; Carroll & Meisch, 1984). Even a mild body weight loss can increase both oral and intravenous drug self-administration (Carroll, et al., 1979; Carroll & Meisch, 1981). Moreover, food deprivation and chronic food restriction not only affect drug taking but can also increase drug seeking. For example, food deprivation lasting 24-48 hours restores extinguished cocaine and heroin seeking using the reinstatement procedure (Shalev, Highfield, Yap, & Shaham, 2000; Tobin, Newman, Quinn, & Shalev, 2009). This acute food-deprivation-induced reinstatement of drug seeking may be in part mediated by leptin, a hormone that is involved in energy balance (Shalev, Yap, & Shaham, 2001). Interestingly, leptin administration has been found to attenuate heroin seeking in food deprived animals, but had little effect on heroin seeking induced by a physical stressor such as footshock or by a priming injection of heroin (Shalev, et al., 2001). These data suggest that reinstatement of drug seeking induced by dietary manipulations is mediated by different neural circuits from those activated by an acute physical stressor or re-exposure to the drug.

Unlike findings in animal models however, acute food deprivation and chronic food restriction differentially affect drug seeking in humans. Although acute food

deprivation will reinstate drug seeking in animals, prolonged food restriction and not acute deprivation is related to increased drug taking in humans (Cheskin, Hess, Henningfield, & Gorelick, 2005; Zacny & de Wit, 1992). Consequently, chronic food restriction may be a more clinically relevant stressor in the study of stress-induced relapse in animal models. Previous research on chronic food restriction in animals has shown that it augments reward and the motivation to seek reward. For example, chronic food restriction in rats significantly augments the reinforcing properties of intra-cranial electrical brains stimulation (Fulton, Woodside, & Shizgal, 2000). In addition, the results of experiments using the conditioned place preference paradigm suggest that chronic food restriction in rats increases the rewarding efficacy of drugs (Stuber, Evans, Higgins, Pu, & Figlewicz, 2002). Similar conclusions have been drawn from experiments in which chronic food restriction has been shown to augment the ability of drugs to lower the threshold for self-stimulation in the lateral-hypothalamus (Carr, 2007). Finally, our laboratory has recently found that 10 days of food restriction reinstates extinguished heroin seeking (Shalev, 2011).

Although in general the results of animal studies of the effects of chronic food restriction on drug taking and or seeking are consistent with the data from human drug users there are two issues that limit their generalizability. One concern is that relapse in humans occurs following a period of abstinence rather than explicit extinction of drug seeking, which is an integral component in the reinstatement procedure. In addition, there is evidence that the neural substrates involved in reinstatement following extinction versus following a period of abstinence are different and have little overlap (Fuchs, Branham, & See, 2006). To address this issue and increase the generalizability of our

results, we investigated drug seeking following a period of abstinence rather than extinction.

A second issue of concern is that most of the previous studies that have investigated the effects of chronic food restriction on drug use have focused on psychostimulant drugs (Carroll & Meisch, 1984), or utilized the electrical brain stimulation paradigm in rats (Carr, 2007, 2011). The generalizability of these results to heroin addiction is difficult since psychostimulant addiction and opiate addiction are likely distinct phenomena with different behavioral and neurobiological factors (Badiani, Belin, Epstein, Calu, & Shaham, 2011).

Studies conducted in both humans and laboratory animals indicate that chronic exposure to psychostimulants may lead to more pronounced deficits in impulse control and cognitive flexibility in comparison to chronic exposure to opiates (Badiani, et al., 2011). Furthermore, rats given unlimited access to psychostimulants develop uncontrolled binge intake, a behavior that is not paralleled in rats given unlimited access to opiates (Badiani, et al., 2011). It has also been suggested that in rats, exposure to cocaine leads to a motivational state that includes both approach and avoidance to the drug, whereas heroin induces a motivational state with only an approach component (Badiani, et al., 2011).

The differences between opiate and psychostimulant addiction extend to differences in drug seeking and relapse. Incubation of drug craving is a phenomenon whereby a longer period of withdrawal or abstinence leads to increased drug seeking induced by drug-associated cues (Grimm, Hope, Wise, & Shaham, 2001; Shalev,

Morales, Hope, Yap, & Shaham, 2001). Although the incubation of drug craving is robust for both cocaine and heroin, the underlying mechanisms mediating this behavior are different. Specifically, endogenous glial cell line-derived neurotrophic factor (GDNF) is involved in the incubation of cocaine craving but not in the incubation of heroin craving (Airavaara et al., 2011; Lu et al., 2009). Finally, the neural circuitry underlying reinstatement of heroin seeking seems to be more widespread in the brain than the circuitry involved in the reinstatement of cocaine seeking (Rogers, Ghee, & See, 2008).

Given the evidence that reinstatement to drug seeking of heroin and stimulant drugs may involve different neural circuits and a similar differentiation of the mechanisms subserving reinstatement and abstinence, in Chapter 1 we present the results of the investigation of the effect of exposure to a prolonged 14-day food restriction period on heroin seeking in abstinent rats. Since these studies showed a robust augmentation of heroin seeking in the food restricted rats, we then began to explore the neuronal mechanisms mediating this effect.

Dopamine (DA), a monoamine neurotransmitter, in the mesolimbic pathway has been implicated in reward and addiction (Kelley & Berridge, 2002; Wise, 2009). The DA neurons in this pathway originate in the ventral tegmental area (VTA) and project to the prefrontal cortex (PFC) and the nucleus accumbens (NAc) (Ungerstedt, 1971). The NAc is a target site of particular interest because both natural rewards and drugs of abuse stimulate DA transmission in this region (Di Chiara, Acquas, Tanda, & Cadoni, 1993). Pharmacologically diverse drugs of abuse all result in increased levels of DA in the NAc through different mechanisms (Bozarth, 1986). For example, opiates, including heroin, indirectly increase extracellular DA in the NAc by disinhibition of DA neurons in the

VTA through the attenuation of GABAergic synapses on these cells (Johnson & North, 1992).

DA is not only released in response to administration of drugs of abuse, but is also related to the motivation to seek reward, whether it is a drug or a natural reinforcer. Studies in humans suggest that DA activity in the mesolimbic pathway is associated with the motivation to obtain a reward and not necessarily with the reward itself (Leyton et al., 2002). In rats, highly palatable foods and drugs of abuse both stimulate DA transmission in the NAc (Di Chiara, 2005). Interestingly, cues that have been previously paired with either a drug or food reward will lead to increased extracellular DA levels in the NAc (Bassareo, De Luca, & Di Chiara, 2007; Bassareo, Musio, & Di Chiara, 2011). DA transmission in the NAc is also strongly implicated in the reinstatement of drug seeking induced by drug re-exposure as well as by drug-associated cues (Shalev, et al., 2002).

Our laboratory has demonstrated that systemic administration of a DA D1 receptor antagonist attenuates acute food-deprivation induced reinstatement of heroin seeking (Tobin, et al., 2009). Unpublished findings also indicate that the food deprivation effect is attenuated by injections of a DA D1 receptor antagonist into brain regions in the mesolimbic DA pathway (Tobin et al., unpublished data), suggesting a critical role for DA transmission in this pathway. However, the involvement of changes in DA release in the effects of chronic food restriction, and more specifically, the role of DA in the augmentation of heroin seeking induced by food restriction has yet to be characterized. Although DA levels in the NAc of food restricted rats increase in response to drug administration (Cadoni, Solinas, Valentini, & Di Chiara, 2003; Rouge-Pont, Marinelli, Le Moal, Simon, & Piazza, 1995), exposure to a previously drug associated context did not

increase NAc DA levels in food restricted rats compared to the sated rats (Stuber, et al., 2002). The results from the latter study should be interpreted with caution however, because they were obtained using the conditioned place preference paradigm, which entails passive drug administration in contrast to the self-administration procedure in which drug delivery is dependent on the behavior of the rat.

As described above, few studies have investigated the effects of chronic food restriction following exposure to opiate drugs. Moreover, none, to date, have measured changes in extracellular DA in the NAc during heroin seeking in food restricted rats. Therefore, given our revised animal model of relapse and the gap in the literature regarding the role of changes in DA release in the effects of chronic food restriction in opiate-drugs-experienced subjects, in Chapter 2 we recorded changes in DA release in the NAc in rats demonstrating augmentation of heroin seeking induced by chronic food restriction.

CHAPTER 1

THE EFFECTS OF CHRONIC FOOD RESTRICTION ON CUE-INDUCED HEROIN SEEKING IN ABSTINENT MALE RATS

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Introduction

There are clear indications that acute food deprivation and chronic food restriction manipulations differentially affect drug- and non-drug-reward seeking. In humans, only prolonged food restriction, and not acute food deprivation, is related to increased drug taking (Cheskin, et al., 2005; Zacny & de Wit, 1992). Similarly, in rats, only prolonged food restriction significantly augmented the reinforcing properties of LHSS (Fulton, et al., 2000).

Thus, more clinically relevant animal models for dietary-induced relapse may need to investigate prolonged periods of food restriction, rather than acute deprivation. Moreover, relapse in humans usually occurs after a period of abstinence rather than explicit extinction of drug seeking, which is an integral part of the reinstatement procedure, thus somewhat diminishing the face validity of the reinstatement procedure (Fuchs, Lasseter, Ramirez, & Xie, 2008). This difference involves more than a simple procedural dissociation, as different neural mechanisms underlie the two behavioral phenomena. For example, compared to cocaine withdrawal, extinction of cocaine self-administration behavior resulted in increases in the GluA1 and GluA2/3 AMPA receptor subunits in the nucleus accumbens shell, a brain region that is critically involved in drug reward and reinstatement of drug seeking (Sutton et al., 2003). Further support for a mechanistic dissociation comes from Fuchs et al. (2006), who have demonstrated that different neural substrates mediate discrete drug-associated-cue-induced reinstatement of cocaine seeking following extinction training versus a similar length abstinence period (Fuchs, et al., 2006).

Here we present a novel model for relapse to heroin seeking following abstinence and 14 days of mild food restriction in the rat. In this revised model, rats are trained to self-administer heroin for a period of 10 days. Following self-administration training, rats are removed from the drug self-administration context and placed in standard shoebox cages in the animal facility for a drug wash-out day, followed by a 14 day period of abstinence. During the abstinence period, half of the rats were food restricted while the other half had free access to food. After the abstinence period rats were brought back to the drug context for a drug seeking test under extinction conditions. We predicted that regardless of food restriction we would see an increase in drug seeking in all the rats following the period of abstinence. Furthermore, we predicted that the food restricted rats would show an augmented drug seeking response compared to the sated rats. Following the demonstration of a robust augmenting effect of food restriction on heroin seeking after 14 days of abstinence, we investigated the importance of the "hunger" state during the test by re-feeding the previously food restricted rats before the drug-seeking test. In addition, to determine if prolonged food restriction is a necessary condition for the augmentation of drug seeking, we manipulated the length of the food restriction period.

Materials & Methods

Subjects

Male Long-Evans rats (n = 92, 325-350 g) purchased from Charles River (St. Constant, Quebec, Canada) were used in the five experiments described below. Rats were allotted 1 week of acclimation prior to surgery, housed in pairs in standard clear shoebox

cages under a reversed 12 h light-dark cycle (lights OFF 9:30 AM; 21˚C). Animals then underwent intravenous (i.v.) catheter implantation surgery and were housed individually in operant conditioning chambers with unrestricted access to food and water, unless otherwise indicated. Following self-administration training, rats were returned to the animal facility and housed individually for the abstinence phase (see details below). Following the abstinence period the rats were brought back to the operant conditioning chambers for the drug-seeking tests.

All rats were treated in accordance with the guidelines of the Canadian Council on Animal Care and approval for all the experimental procedures was granted by the Concordia University Animal Research Ethics Committee.

Surgical procedures

All animals were anesthetized with ketamine + xylazine (10 + 100 mg/kg, i.p) before i.v. catheterization. The jugular vein was carefully isolated and a Silastic catheter (Dow Corning, Midland, MI, USA) was inserted 3 cm into the vein and secured in place with silk sutures. The catheter was then subcutaneously threaded to the top of the skull and attached to a modified 22-gauge cannula (Plastics One Industries, Roanoke, VA, USA). The cannula was finally mounted in place with jewelers' screws and dental cement. Following surgery, rats were administered penicillin (450 000 IU/rat, s.c.) and analgesic buprenorphine (10 μg/kg, s.c.), and kept on a heating pad until sufficient recovery. Animals were the given 1 week to recover prior to self-administration training. Throughout self-administration training catheters were flushed daily with heparin and

gentamicin in sterile saline (7.5 IU + 12.0 μg per day per rat) to prevent catheter blockage.

Apparatus

Experiments were conducted in operant conditioning chambers (Med Associates Inc., St. Albans, Vermont, USA; 32.0 cm × 24.0 cm × 25.0 cm), placed in individual sound-attenuated cubicles. Each box comprised 2 levers located 5 cm above the grid floor. Responses on the "active", retractable, lever activated the infusion pump (Med Associates), whereas responses on the "inactive", nonretractable, lever were recorded but had no programmable consequences (Maric, Sedki, Ronfard, Chafetz, & Shalev, 2011).

Procedure

Different cohorts of rats were used for each of the five experiments, which followed a similar general procedure. All experiments consisted of three phases: heroin self-administration training in operant conditioning chambers, an abstinence phase in the home cage during which some rats were food restricted, and a testing phase in the operant conditioning chambers. Timelines for the different experiments are presented in Figure 1.

Training

Following a 24 h habituation period to the operant conditioning chambers, heroin self-administration training was conducted for 10 days. Rats were given three-3 h training sessions per day separated by a 3 h period under a fixed ratio 1 (FR-1) schedule

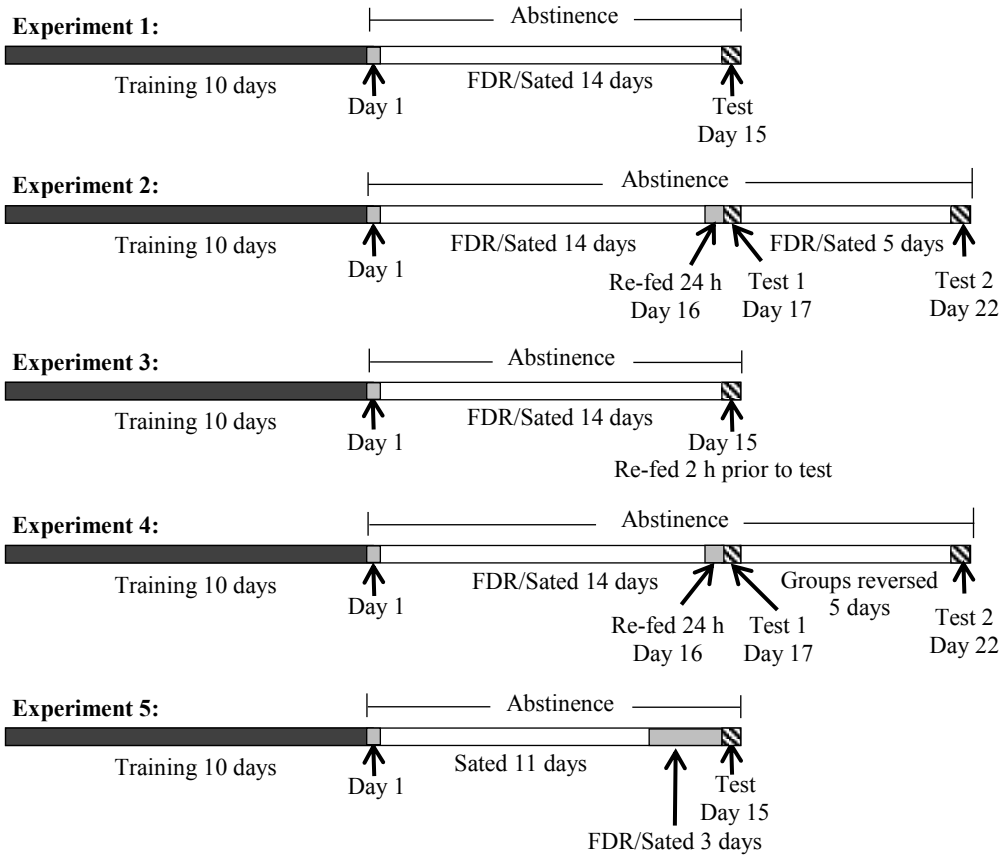

Figure 1 Overview of timelines for Experiments 1 through 5 in Chapter 1.

with 20 s timeout. The initial session began shortly after the onset of the dark phase and was marked by the insertion of the active lever, illumination of a houselight, and activation of a cue-light/tone (2.9 kHz; 10 dB above background level) complex above the active lever, which remained active for 30 s or until the active lever was pressed. Responding on the active lever resulted in the delivery of 0.1 mg/kg of heroin (diacetylmorphine HCl; provided by the National Institute for Drug Abuse, Research Triangle Park, NC, USA) in 0.13 ml infusion over 12 seconds and the initiation of a 20 s timeout, during which the houselight was turned off, the cue-light/tone complex remained on, and additional responses on the active lever were recorded but not reinforced. At the end of each 3 h session the active lever retracted and the houselight turned off.

Experiment 1: The effect of 14 days of food restriction on heroin seeking in abstinent rats

Following self-administration training, rats were given a drug washout day where they were individually housed in the animal facility with unrestricted access to food and water. The next day, rats were divided into 2 groups: food restricted (FDR) or sated. Groups were matched for average number of infusions, active lever responses and body weight during the last 5 days of training. The FDR rats had their food removed, and were fed ~15 g of rat chow at 1:30 PM; this amount was adjusted daily to bring the FDR rats' body weight to approximately 75-80% of the sated rats'.

On abstinence day 15, which was the 14th day of food restriction (FDR 14), rats were brought back to the operant conditioning chambers. FDR rats had empty food hoppers in their operant conditioning chambers. Testing took place under extinction

conditions over a 3 h session. Active lever responses resulted in similar consequences as in training but in the absence of heroin infusions.

Experiment 2: The effects of a 24 h re-feeding period (following 14-day food restriction) and 5-day re-food-restriction on heroin seeking in abstinent rats

The significance of the state of hunger during the drug-seeking test for the augmentation effect seen in Experiment 1 was investigated here. Rats were tested twice, once under sated conditions and a second time following a re-food-restriction (re-FDR) period.

As in Experiment 1, rats were given a washout day and separated into FDR and sated groups for the abstinence period in the animal facility. Following 14 days of food restriction, FDR rats were given unrestricted access to food for 24 hours (on abstinence day 16) prior to being tested for drug seeking on abstinence day 17. Rats were re-fed on abstinence day 16 to ensure the same duration of food restriction as in Experiment 1. All rats were given unrestricted access to food and water during the test. Testing was conducted under extinction conditions as in Experiment 1, except that the duration of the tests was shortened to 1 h to minimize extinction over repeated testing. Following Test 1, rats were returned to the animal facility, where the previously FDR rats were re-restricted from abstinence day 18 to the second test on abstinence day 22. Test 2 took place on abstinence day 22 under extinction conditions similar to the Test 1.

Experiment 3: The effects of a 2 h re-feeding period on heroin seeking in abstinent rats

Over a 24 h re-feeding period, food digestion and metabolic changes could contribute to the results observed in Experiment 2. Therefore, in order to examine the

effect of the hunger state *per se*, rats were re-fed 2 h prior to the drug seeking test, allowing for minimal digestion, although considerable amounts of food were consumed.

The FDR and sated groups of rats were treated as described in Experiment 1. On abstinence day 15 (or FDR 14), FDR rats were given unrestricted access to food for 2 h in their home cages prior to the testing, which was conducted, under extinction conditions, over a 1 h as in experiment 2. All rats were given unrestricted access to food and water during the test.

Experiment 4: The effects of a 24 h re-feeding period, and 5-day food restriction on heroin seeking in abstinent rats

The purpose of this experiment was to replicate the finding in Experiment 2, and in addition to explore the effects of a short-term food restriction on the augmentation of heroin seeking in abstinent rats.

As in Experiment 1 and 2, following training rats were returned to the animal facility for a washout day, separated into FDR and sated groups, and food restricted for 14 days. On abstinence day 16, FDR rats were re-fed and tested, under extinction conditions, the following day (abstinence day 17), over a 1 h session, similar to Experiment 2 (Test 1). Following Test 1, rats were returned to the animal facility, and the previously FDR rats were allowed unrestricted access to food for 5 days whereas the previously sated rats were now food restricted for 5 days. On abstinence-day 22 rats were tested again, under extinction conditions (Test 2).

Experiment 5: The effect of a 3-day food restriction period on heroin seeking in abstinent rats

In order to avoid the possible confounding effects of the repeated tests in Experiment 4, rats were exposed to only one drug-seeking test following a short food restriction period.

After the drug washout day in the animal facility, rats had 11 additional days of unrestricted access to food and water prior to being separated into a sated group and an FDR group that was food restricted for 3 days. Testing took place on abstinence day 15 (FDR 3 for food restricted rats), and consisted of a 1 h session under extinction conditions.

Statistical Analyses

For Experiments 1, 4 and 5 active and inactive lever responses during the test session were analyzed separately using independent samples, two-tailed *t*-test to compare the means of the FDR and sated groups. For Experiments 2 and 3 the number of responses made on the active and inactive levers during testing were analyzed separately using repeated measures ANOVA with the between subjects factor of *food restriction* (FDR, Sated) and the within subjects factor of *test day* (Test 1, Test 2). Statistically significant interactions were followed by post hoc (Fisher's LSD) tests. Significant results are reported for $p \leq 0.05$.

Results

Rats in all experiments acquired reliable heroin self-administration behavior. The mean ± SEM number of infusions, and active and inactive lever responding made on the last day of heroin self-administration training, for each experiment, are shown in Table 1. Mean ± SEM body weights of the FDR and sated groups for each experiment are detailed in Table 2. In addition, in representative groups of rats, the average 24 h food intake for the sated rats over the abstinence period was 30.39 ± 0.89 g, while the FDR rats were fed, on average, 14.21 ± 0.13 g of chow per day over the same period.

Experiment 1: The effect of 14 days of food restriction on heroin seeking in abstinent rats

The average body weights for the rats throughout the experiment are presented in Figure 2.

On test day, a 14-day food restriction period resulted in a significant increase in responding on the active lever, previously associated with heroin administration, compared to sated rats, $t(16) = 2.30$, $p = 0.03$ (Figure 3). There were no significant differences in the number of inactive lever responses between groups. The individual distribution of responses on the active and inactive levers during the test session in the FDR and sated rats is presented in Figure 4.

Table 1. Mean ± SEM of the number of infusions taken, and the number of active and inactive lever responses made on the last training day (9 h) in each experiment.

	Infusions	Active lever	Inactive lever
Exp. 1	34.72 ± 3.66	89.11 ± 16.47	12.61 ± 4.12
Exp. 2	41.65 ± 6.23	145.05 ± 37.89	11.10 ± 3.19
Exp. 3	47.58 ± 4.74	150.42 ± 26.44	6.32 ± 1.99
Exp. 4	44.00 ± 4.56	145.72 ± 27.69	6.39 ± 1.63
Exp. 5	40.16 ± 6.81	173.48 ± 62.97	3.44 ± 0.75

Table 2. Mean ± SEM body weight (g) in the sated and food restricted (FDR) groups. Numbers in parentheses indicate percent body weight compared to the sated group, and compared to own body weight on the washout day, respectively.

	Group	FDR 14-day	Re-feeding 24 h	Re-feeding 2 h	FDR 5-day	FDR 3-day
Exp. 1	Sated n = 8	464.88 ± 16.60	-	-	-	-
	FDR n = 10	336.30 ± 9.77 (72%, 89%)	-	-	-	-
Exp. 2	Sated n = 10	514.7 ± 13.35	527.20 ± 14.35	-	-	-
	FDR n = 10	388.40 ± 6.54 (75%, 90%)	424.10 ± 7.61 (80%, 98%)	-	-	-
Exp. 3	Sated n = 12	454.25 ± 11.96	-	459.17 ± 12.07	-	-
	FDR n = 13	352.85 ± 5.27 (78%, 93%)	-	375.46 ± 5.03 (82%, 99%)	-	-
Exp. 4	Sated n = 10	462.30 ± 11.48	474.60 ± 10.08	-	456.30 ± 6.93* (101%, 118%)	-
	FDR n = 9	369.33 ± 13.10 (80%, 94%)	405.22 ± 9.93 (85%, 103%)	-	452.78 ± 4.09#	-
Exp. 5	Sated n = 8	-	-	-	-	482.63 ± 14.45
	FDR n = 10	-	-	-	-	451.00 ± 12.63 (93%, 112%)

* Following a 5-day food restriction period

Following a 6-day period of unrestricted access to food

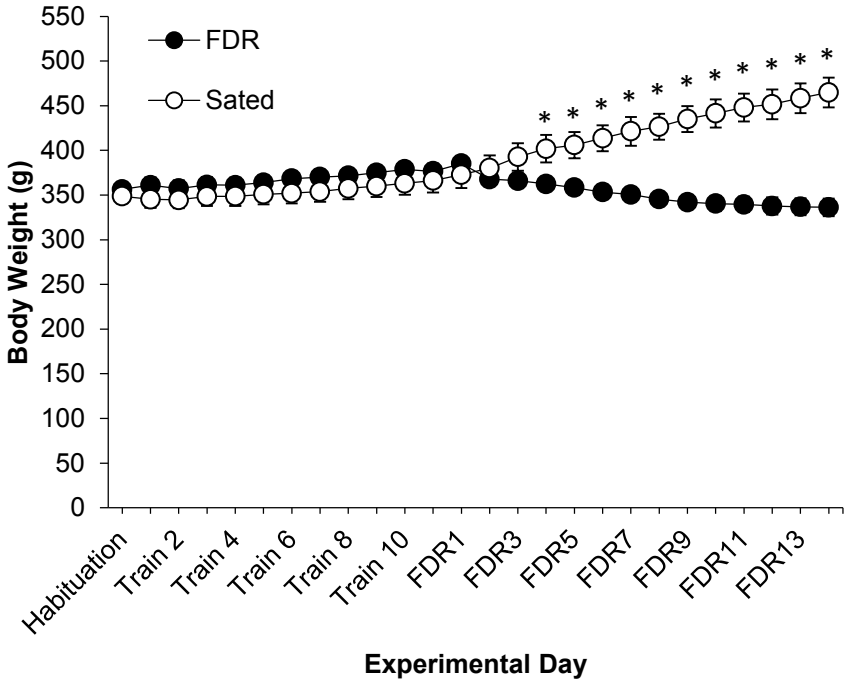

Figure 2 Mean (± SEM) body weights of all rats over the course of Experiment 1 in the food restricted (FDR, n=10) and sated (n=8) groups. * $p < 0.05$ compared to FDR group.

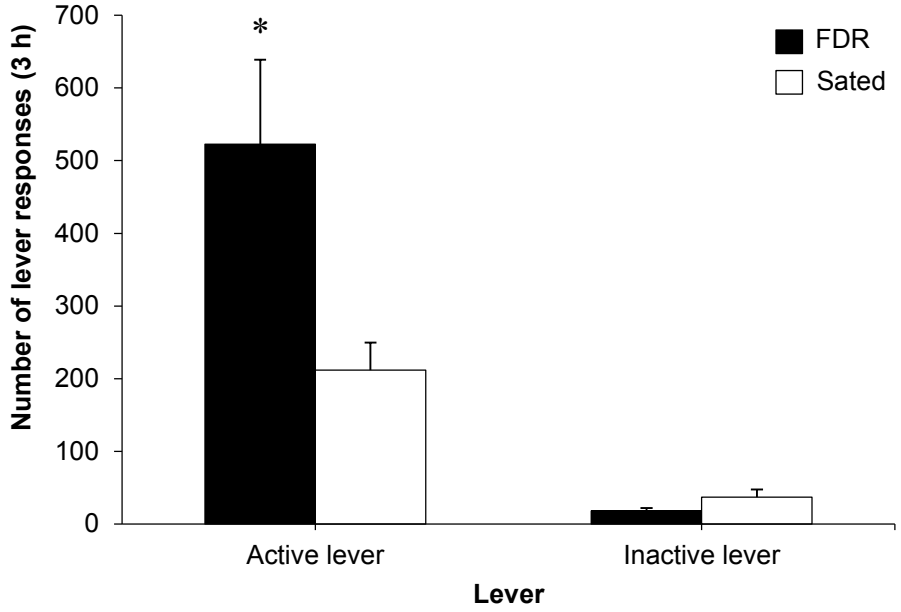

Figure 3 The effect of 14 days of food restriction and abstinence on heroin seeking (Experiment 1). Data are mean (+ SEM) number of responses made on the active and inactive levers on the test day (FDR 14) in the FDR (n=10) and sated groups (n=8). * $p <$ 0.05 compared to the sated group.

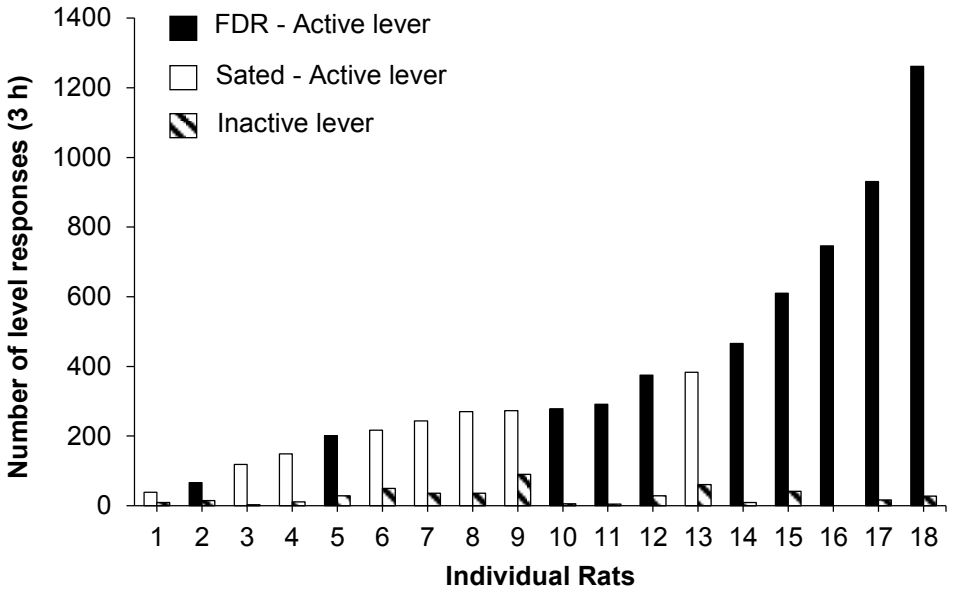

Figure 4 The effect of 14 days of food restriction and abstinence on heroin seeking (Experiment 1). Data are the number responses made by each rat on the active and inactive levers on the test day (food restriction day 14).

Experiment 2: The effects of 24 h re-feeding (following 14-day FDR) and 5-day re-food-restriction on heroin seeking in abstinent rats

Re-feeding 24 h prior to the 1st test session (Test 1) eliminated the food restriction-induced augmentation of heroin seeking reported in experiment 1. Following re-restriction for 5 additional days prior to Test 2, however, the FDR rats had a higher rate of responding on the active lever than sated rats (Figure 5). Repeated measures ANOVA revealed significant effects for *food restriction* ($F_{1,18} = 5.07$, $p = 0.04$) and *food restriction* × *test day* ($F_{1,18} = 6.09$, $p = 0.02$), but *test day* effect was not significant. Post hoc tests revealed a significant difference between the FDR and sated rats on Test 2 ($p < 0.05$). There were no significant differences between the groups in inactive lever responding on either test.

Experiment 3: The effects of 2 h re-feeding on heroin seeking in abstinent rats

There was no statistically significant difference in the number of active lever responses made by the FDR rats that were allowed 2 h of unrestricted access to food prior to the heroin-seeking test compared to the sated group (Figure 6). There were no significant differences in inactive lever responses between the groups.

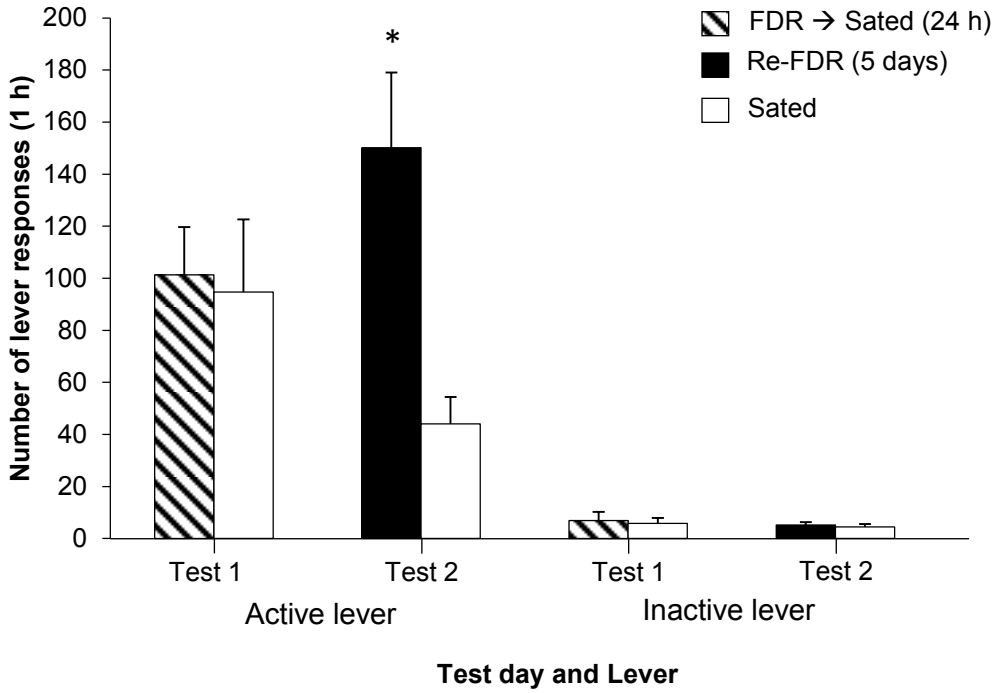

Figure 5 Heroin seeking following 14 days of food restriction and re-feeding (24 h) in abstinent rats (Experiment 2). Data are mean (+SEM) number responses made on the active and inactive levers on Test 1 (abstinence-day 17) following 14 days of food restriction and one day of unrestricted access to food (FDR → Sated 24 h; n=10), compared to continuously sated rats (n=10), and following 5 days of re-FDR (Test 2). * $p < 0.05$ compared to the sated group.

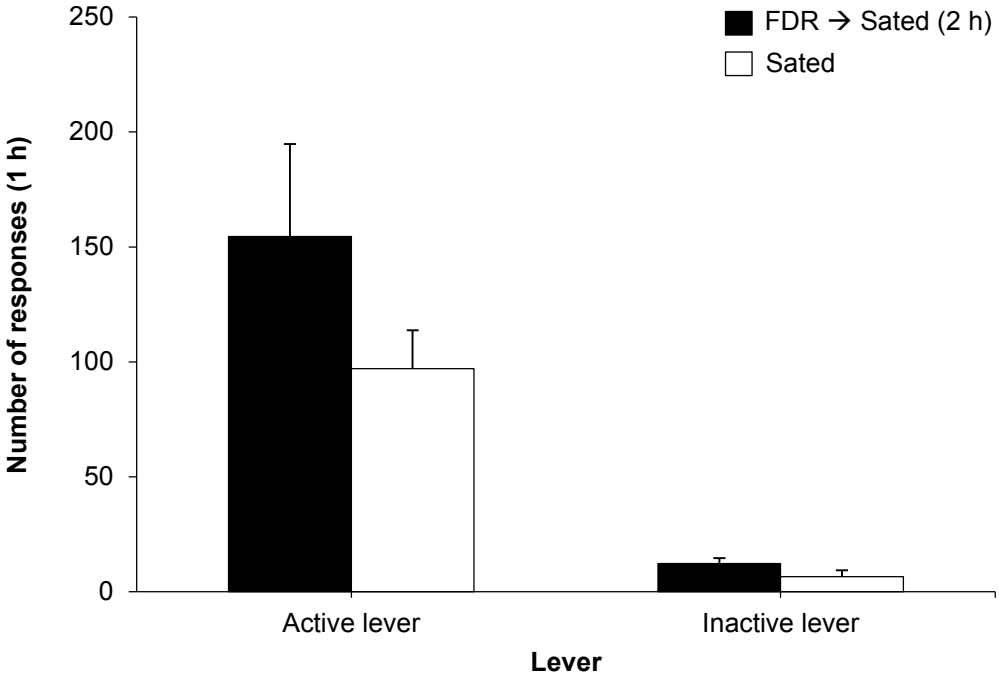

Figure 6 The effects of 2 h re-feeding on heroin seeking following 14 days of food restriction in abstinent rats (Experiment 3). Data are the mean (+SEM) number responses made on the active and inactive levers on the test day (FDR 14) in the FDR-re-fed (FDR → Sated, n=13) and sated groups (n=12).

Experiment 4: The effects of 24 h re-feeding and 5-day food restriction on heroin seeking in abstinent rats

No significant differences between the groups were found in active lever responses when previously FDR rats had been re-fed for 24 h (Test 1) or when previously sated rats had been food restricted for 5 days (Test 2). The number of active lever responding in Test 2 was lower than Test 1 in both groups, suggesting an extinction of responding over repeated tests, but the difference was not statistically significant (*test day*: $F_{1,8} = 8.08$, $p < 0.07$). The effect of *food restriction* and the interaction effect for *food restriction* × *test day* were not significant (Figure 7). The number of inactive lever responses were low compared to active lever responses, and were lower on Test 2 than on Test 1. Repeated measures ANOVA performed on the inactive lever data revealed a significant effect for *test day* ($F_{1,8} = 8.08$, $p = 0.02$), but not for *food restriction* or *food restriction* × *test day* interaction.

Experiment 5: The effect of 3-day FDR on heroin seeking in abstinent rats

Following 11 days of abstinence in the animal colony with both groups sated, 3 days of food restriction were not sufficient to cause a significant increase in active lever responses during tests for heroin seeking under extinction conditions compared to sated rats (Figure 8). There were no significant differences between the groups in inactive lever responding.

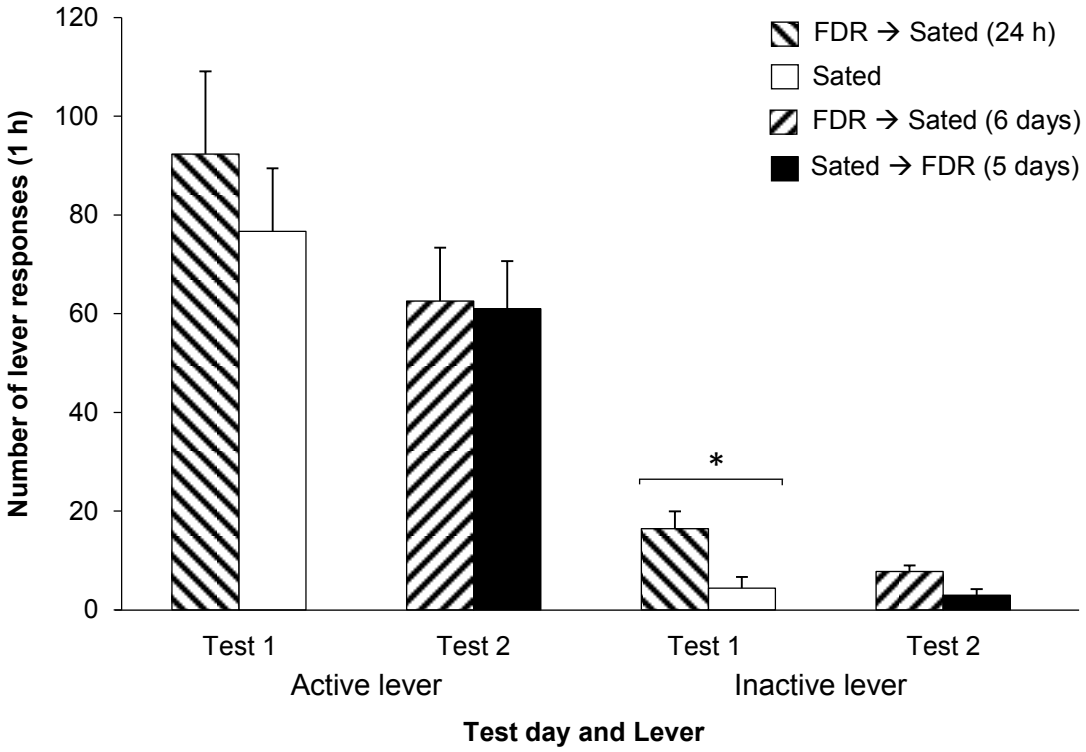

Figure 7 Heroin seeking following re-feeding, and after a short (5 days) food restriction period in abstinent rats (Experiment 4). Data are mean (+SEM) number responses made on the active and inactive levers on Test 1 (abstinence-day 17) following 14 days of food restriction and one day of unrestricted access to food (FDR → Sated 24 h; n=9), compared to continuously sated rats (Sated, n=10), and following 5 additional days of unrestricted access to food (FDR → Sated 6 days) compared to 5 days of food restriction in the previously sated rats (Sated → FDR 5 days) on Test 2. * $p < 0.05$ compared to Test 2.

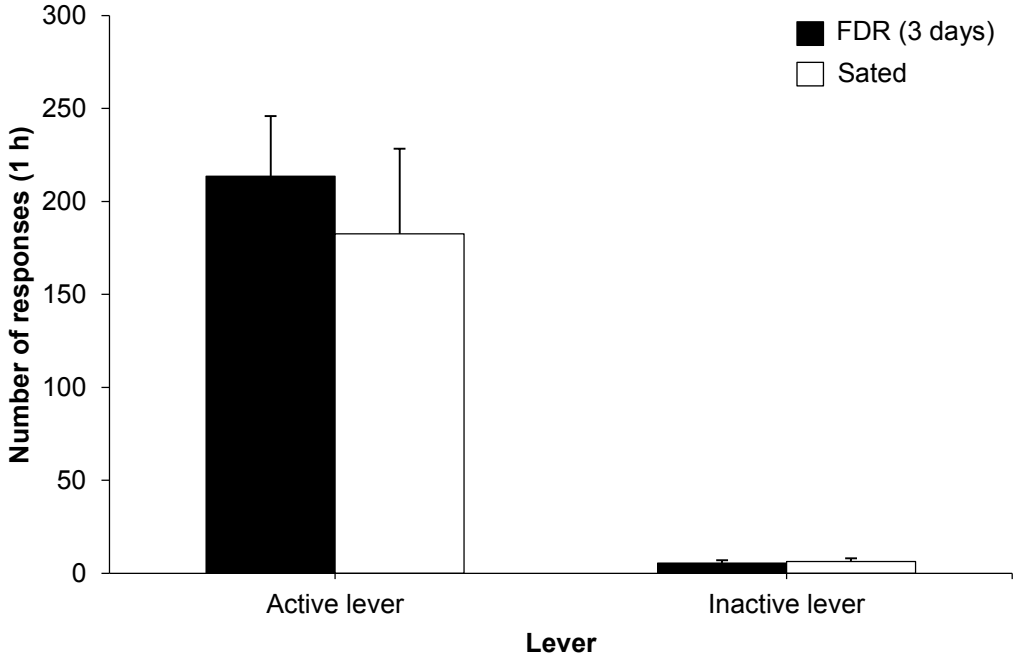

Figure 8 The effect of a short (3 days) food restriction period on heroin seeking in abstinent rats (Experiment 5). Data are the mean (+SEM) number responses made on the active and inactive levers on the test day (abstinence day 15) in the FDR (FDR 3, n=10) and sated (n=8) groups.

Summary

As expected, all the rats that had experienced a period of abstinence showed robust heroin seeking when re-exposed to the drug-associated environment and cues. The major finding in this study, however, is that the chronically food restricted rats showed dramatically higher levels (>250%) of heroin seeking compared to the sated rats. Food restriction-induced augmentation of drug seeking proved to be a reliable effect, with at least 60% of the subjects in Experiment 1 producing more than 350 responses during the 3 h test, compared to only 12.5% of the sated rats. Interestingly, re-feeding the FDR rats for a 24 h period completely eliminated the food restriction effect. A 2 h re-feeding period was sufficient to reduce the augmentation of heroin seeking in FDR rats such that the difference in bar press rates between FDR and sated rats was no longer statistically significant. These data suggest that a concurrent state of hunger is necessary to augment to heroin seeking in food restricted rats. In addition, the results of this series of studies suggest that chronic food restriction is required to increase heroin seeking because 3 days of food restriction at the end of 14 days of abstinence did not increase heroin seeking. In contrast, food restricting rats for 5 days following 24 h of re-feeding did induce a greater level of drug seeking in previously food restricted than in to the sated rats (Experiment 2). The implications of these findings are elaborated upon the general discussion.

CHAPTER 2

CHANGES IN DOPAMINE IN THE NUCLEUS ACCUMBENS SHELL ASSOCIATED WITH THE AUGMENTATION OF HEROIN SEEKING INDUCED BY FOOD RESTRICTION

Tracey D'Cunha, Laurie Hamel, Firas Sedki, Uri Shalev

Introduction

Consumption or anticipation of drugs of abuse, natural rewards, as well as stressors all share the common outcome of increasing DA levels in the mesolimbic pathway, which has been implicated in reward and addiction (Bozarth, 1986; Di Chiara, et al., 1993; Kalivas & Duffy, 1995; Kelley & Berridge, 2002; Wise, 2009). Moreover, DA transmission in the NAc is not only affected by the consumption of rewards, but has also been implicated in the motivation to seek reward, both for natural rewards and drugs. Thus, cues that have been previously paired with the availability of a reward can also stimulate DA release (Bassareo, et al., 2007; Bassareo, et al., 2011; Schultz, Dayan, & Montague, 1997). DA transmission in the NAc is also strongly implicated in the reinstatement of drug seeking triggered by drug re-exposure as well as by drug-associated cues (Shalev, et al., 2002). Blocking DA receptors, with a DA D1 receptor antagonist, in the NAc attenuates cue-induced reinstatement of heroin seeking (Bossert, Poles, Wihbey, Koya, & Shaham, 2007). In addition, administration of a DA D1 receptor antagonist will also attenuate food-deprivation induced reinstatement of heroin seeking (Tobin, et al., 2009). However, the role of DA in the augmentation of heroin seeking induced by food restriction has yet to be characterized.

The NAc, a major target site for the mesolimbic DA pathway, can be divided into two main subregions: the NAc core and the NAc shell. Studies indicate a definite dichotomy between the projections of the NAc shell versus the core (Ikemoto, 2007). Both the shell and the core project to the ventral pallidum (VP), a structure that is part of the basal ganglia and implicated in drug addiction and motor control (Alexander & Crutcher, 1990); however, the core projects primarily to the dorsolateral part of the VP,

whereas the shell projects primarily to the ventromedial part of the VP (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). In addition, the NAc shell but not the core, projects to the extended amygdala, and is thought to be a transition area between the striatal complex and the extended amygdala (Heimer, et al., 1991).

Several reports indicate that the NAc shell and core may play different roles in mediating drug related behaviors (Ikemoto, 2007). Administration of a DA D1 receptor antagonist into the NAc shell attenuates context-induced reinstatement of heroin seeking, whereas this antagonist has no effect in the NAc core. In contrast, administration of the same antagonist into the NAc core attenuated discrete-cue-induced reinstatement of heroin seeking, but was ineffective when administered into the NAc shell (Bossert, et al., 2007). Moreover, inactivation of the NAc core abolished conditioned cue-induced reinstatement of cocaine seeking, whereas inactivation of the NAc shell had no effect (Fuchs, Evans, Parker & See, 2004).

DA transmission in the NAc shell is postulated to be more important for drug reward than the NAc core (Ikemoto, 2007). In particular, extracellular DA is elevated more in the shell than in the core in response to intravenous administration of a variety of drugs, such as amphetamine, cocaine, or morphine (Pontieri, Tanda, & Di Chiara, 1995). Furthermore, blockade of DA receptors in the shell decreases specifically the reinforcing efficacy of cocaine and not food, as measured by a progressive ratio schedule of reinforcement, while blockade of DA receptors in the core indiscriminately decreases cocaine self-administration as well as food self-administration (Bari & Pierce, 2005).

DA in the shell is modulated not only by drug administration but also by cues previously paired with drug availability. For example, cues previously associated to drugs elevate DA in the NAc shell but not the NAc core. Conversely, cues previously associated with food availability elevate DA in the NAc core but not the NAc shell (Bassareo, et al., 2011). These dissociative effects might be explained by activation of different meso-striatal projections by drugs and natural rewards. The medial NAc shell receives the majority of its projections from the posteromedial VTA, whereas the NAc core and lateral shell receive projections from the anteromedial VTA (Ikemoto, 2007). Interestingly, it was demonstrated that the rewarding effects of opiates, as measured by the conditioned place preference paradigm, are mediated by the posterior but not anterior VTA; however, the rewarding effects of food were not assessed in this study (Zangen, Ikemoto, Zadina, & Wise, 2002). Collectively, these results suggest that DA in the NAc core may mediate the reinforcing properties of general rewards, such as food, but that DA transmission in the NAc shell may be more selective for drug reward.

In this chapter we investigated the role of changes in DA release in the NAc shell in the augmentation of heroin seeking induced by food restriction in the novel animal model of relapse described in Chapter 1. As in Chapter 1, rats were trained to self-administer heroin for a period of 10 days. Following self-administration training, rats were moved to the animal colony for a drug wash-out day followed by a 14 day period of abstinence. During this period of abstinence rats were assigned to two groups: food restricted and sated. After the abstinence period, rats were brought back to the drug-associated context for a drug seeking test under extinction conditions. In addition, during this drug seeking test *in vivo* microdialysis was conducted to determine changes in

extracellular DA levels in the NAc shell. We predicted that we would see a robust augmentation of heroin seeking induced by food restriction following a period of abstinence, as was demonstrated in Chapter 1 Experiment 1. We also predicted that increases in extracellular DA levels would be observed in all the rats regardless of food restriction when they were returned to the drug context but that this would be greater in the food restricted group.

Materials & Methods

Subjects

Male Long Evans rats (n = 12, 325-350 g) were purchased from Charles River (St. Constant, Quebec, Canada). Rats were acclimated to the animal facility for a week and were housed in pairs until surgery in standard clear shoebox cages under a reversed 12 h light-dark cycle (lights OFF 9:30 AM) with the temperature at approximately 21˚C. Following recovery from surgery rats were housed individually in operant conditioning training chambers with unrestricted access to food and water.

As in Experiment 1, Chapter 1, rats were returned to the animal facility for the duration of the abstinence and food restriction phase. Prior to *in vivo* microdialysis sampling, rats were placed in clear Plexiglass chambers with a grid floor in order to collect baseline dialysate samples prior to the drug seeking test (see below). Following baseline microdialysis collection rats were transferred back to the operant conditioning chambers for the drug seeking test.

All rats were treated in accordance with the guidelines of the Canadian Council on Animal Care and approval for all the experimental procedures was granted by the Concordia University Animal Research Ethics Committee.

Surgical procedures

All rats underwent IV catheterization under anaesthesia as described in Chapter 1. During the same surgery rats were also implanted with a guide cannula for *in vivo* microdialysis (Bioanalytical Systems Inc., West Lafayette, IN, USA) targeting the nucleus accumbens shell (AP: +1.8 mm, ML: ±1.0 mm, DV: -6.2 mm, relative to Bregma). The guide cannula was mounted beside the modified catheter cannula on the skull using jewelers' screws and dental cement. Cannula placements were counter-balanced between the right and left hemispheres. Following surgery, rats were given penicillin (450 000 IU/rat, s.c.), and the analgesic buprenorphine (600 μg/rat, s.c.), as well as isothermal heating until recovery from anaesthesia. Throughout self-administration training catheters were flushed daily with heparin and gentamicin in sterile saline (7.5 IU + 12.0 μg per day per rat) to prevent catheter blockage.

Apparatus

Self-administration training and the drug seeking tests were conducted in operant conditioning chambers (Med Associates Inc., St. Albans, Vermont, USA) as described in Chapter 1. Prior to drug seeking tests rats were placed in a clear Plexiglass chamber with a grid floor in the animal facility for baseline microdialysis sampling (30.0 cm × 28.0 cm × 25.0 cm).

Procedure

As in Chapter 1 there were three phases in the experiment: heroin self-administration training in the operant conditioning chambers, an abstinence phase in the home cages in the animal facility during which some of the rats were food restricted, and a testing phase in the operant conditioning chambers. A timeline of the experiment is presented in Figure 9.

Training

Heroin self-administration training was as described in Chapter 1. Briefly, following a 24h habituation period to the operant conditioning chambers, rats were trained to self-administer heroin 0.1 mg/kg/infusion) over a period of 10 days.

Abstinence and Food Restriction

Following heroin self-administration rats were transferred to the animal facility and housed individually in standard shoebox cages with unrestricted food and water for a drug washout day. Rats were matched for number of infusions, active lever responses and body weight during the last 5 days of training and divided into 2 groups: food restricted (FDR) and sated. FDR rats were fed approximately 15 g of food daily at 1:30 PM. This ration was adjusted daily to bring the body weight of the FDR group to approximately 75-80% of the sated rats'.

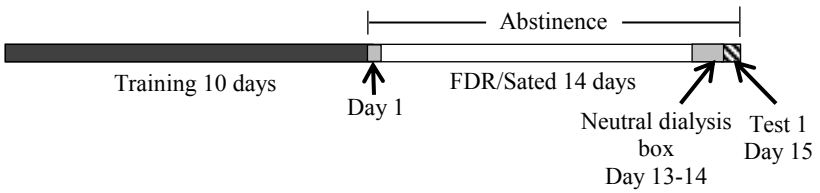

Figure 9 Overview of timeline for the experiment in Chapter 2.

In Vivo Microdialysis & Testing Phase

On abstinence day 13 (or food restriction day 12, FDR 12) rats were transferred to neutral Plexiglass chambers in the animal facility. Rats were allowed unrestricted access to water and food unless they were food restricted. FDR rats received their daily allotment of food in these chambers. On abstinence day 14 (FDR 13) rats were anesthetized lightly with isoflurane in the animal facility in order to lower *in vivo* microdialysis probes into the NAc. Once the rat was immobile, cannula obturators were removed and microdialysis probes with a 2.0 mm active membrane (Bioanalytical Systems Inc., West Lafayette, IN, USA) were inserted into the guide cannula targeting the nucleus accumbens shell. To stabilize the probe/brain interface, probes were perfused with artificial cerebrospinal fluid (aCSF; 145mM Na^+, 2.7mM K^+, 1.22mM Ca^{2+}, 1.0mM Mg^{2+}, 150mM Cl^-, 2mM Na_2PO_4, pH 7.4 ±0.1) at a flow rate of 1.0 µl/min for approximately 1 h. The flow rate was then lowered to 0.2 µl/min overnight. On abstinence day 15 (FDR 14) the flow rate of the microdialysis pump was increased by to 1.0 µl/min approximately 1 h prior to baseline sampling.

At approximately 8:10 AM baseline collection started, and dialysate samples were collected every 10 minutes. At 9:20 AM rats were transported from the animal facility to the room with the operant conditioning chambers. Microdialysis pumps were plugged into a battery pack on a cart so that the flow rate and sampling was never interrupted. Rats were transferred back into the operant conditioning chambers for the drug-seeking test. Prior to the initiation of the test, rats were habituated to the chamber for about 10 minutes. Testing took place under extinction conditions over a 3 h session, and dialysate samples were collected at 10 min intervals. Active lever responses resulted in the same

consequences as in training (i.e., the initiation of a 20 s timeout, during which the houselight was turned off, and activation of the cue-light/tone), with the exception that no heroin infusions occurred.

Analytical Chemistry

DA and its metabolites were separated from other chemical species in the dialysate samples using high performance liquid chromatography (HPLC) and quantified using electrochemical detection (ED) as described in (Hernandez, Rajabi, Stewart, Arvanitogiannis, & Shizgal, 2008). Dialysate samples were loaded through manual injection ports (Rheodyne 7125; Rheodyne LLC, Rhonert Park, CA; 20µl loop) into a reverse-phase column (15cm × 0.46cm Spherisorb-ODS, 5µm; Higgins Analytical, Mountain View, CA). Following separation in the column the sample passed through dual-channel ESA (Chelmsford, MA) coulometric detectors (Coulochem 5100, with a model 5011 analytical cell), which were connected to a computer. The detectors were set to reduce DA in one channel and to oxidize DA's metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in the other channel. Standard samples of solutions containing known concentrations of DA and its metabolites were used to calibrate the equipment. Waters 515 HPLC pumps (Lachine, Quebec, Canada) were used to circulate the mobile phase (19% acetonitrile, 40mg 0.076M SDS, 0.1M EDTA, 0.058M $NaPO_4$, 0.03M citric acid, pH 3.35) at a flow rate of 1.2mL/min. EZChrom Chromatography Data System (Scientific Software Inc., San Ramon, CA) was used to analyze and integrate the data obtained for DA, DOPAC and HVA. Two HPLC-ED systems were used in parallel to analyze the samples, but all dialysate samples from a given rat were analyzed on the same system.

Histology

After the completion of the experiment rats were euthanized with carbon dioxide gas and decapitated. Brains were fixed with a 4% paraformaldehyde solution for a week before being sliced in 40 μm coronal sections with a cryostat. Slices were then stained with cresyl violet and cannula and probe locations were determined under a microscope with reference to the brain atlas of Paxinos and Watson (2005).

Statistical Analyses

The number of responses made on the active and inactive levers during the test session were analyzed separately using independent samples two-tailed *t*-test to compare the means of the FDR and sated groups. Baseline absolute concentrations (pg/μl) of DA and its metabolites were analyzed using repeated measures ANOVA with the between subjects factor of *food restriction* (FDR, Sated) and the within subjects factor of *time* (3 baselines samples preceding the drug seeking test). To assess the effect of food restriction on DA transmission in the NAc shell, baseline levels of DA, DOPAC and HVA were determined by averaging the 3 samples collected prior to the move to the operant conditioning chambers for each rat and then converting the values of all test session samples to a percentage of baseline. Changes from baseline of DA, DOPAC and HVA were analyzed separately using repeated measures ANOVA with the between subjects factor of *food restriction* (FDR, Sated) and the within subjects factor of *time* (baseline average, context change and test samples 1-17). Statistically significant interactions were followed by post hoc (Fisher's LSD) tests. Significant results are reported for $p \leq 0.05$.

Results

Twelve rats were trained to self-administer heroin and subsequently tested for drug seeking. However, 3 rats pulled their probes out during the night prior to the test session, and the data of 3 rats were lost due to technical problems with the analysis of the dialysate samples in the HPLC-ED. Thus, the final analyses included 6 rats, 2 rats in the sated group and 4 rats in the FDR group. Only rats with correct histological placements in the nucleus accumbens shell were included in the analyses (Figure 10). All rats acquired reliable heroin self-administration behavior. The mean (±SEM) number of infusions on the last day of training was 39.33 (±12.55). The mean (±SEM) number of responses on the active and inactive levers on the last day of training was 113.83 (±55.02) and 5.17 (±3.44), respectively. On test day (FDR 14) the mean ± SEM body weight for the FDR rats (n = 4) was 302.5 ± 7.66 g, which was approximately 76% of the sated rats' mean body weight (398 ± 4 g; n = 2), or approximately 87% of their body weight on FDR 1 (See Figure 11 for mean body weight throughout the experiment).

Heroin seeking following 14 days of food restriction

On abstinence day 15 (FDR 14), food restricted rats demonstrated a significant increase in heroin seeking compared to the sated rats, $t(4) = 2.722$, $p = 0.05$ (Figure 12). There were no significant differences in the number of inactive lever responses between groups. Figure 13 shows active lever responding for the FDR and sated rats in 10-min time intervals across the entire 3 h drug seeking test.

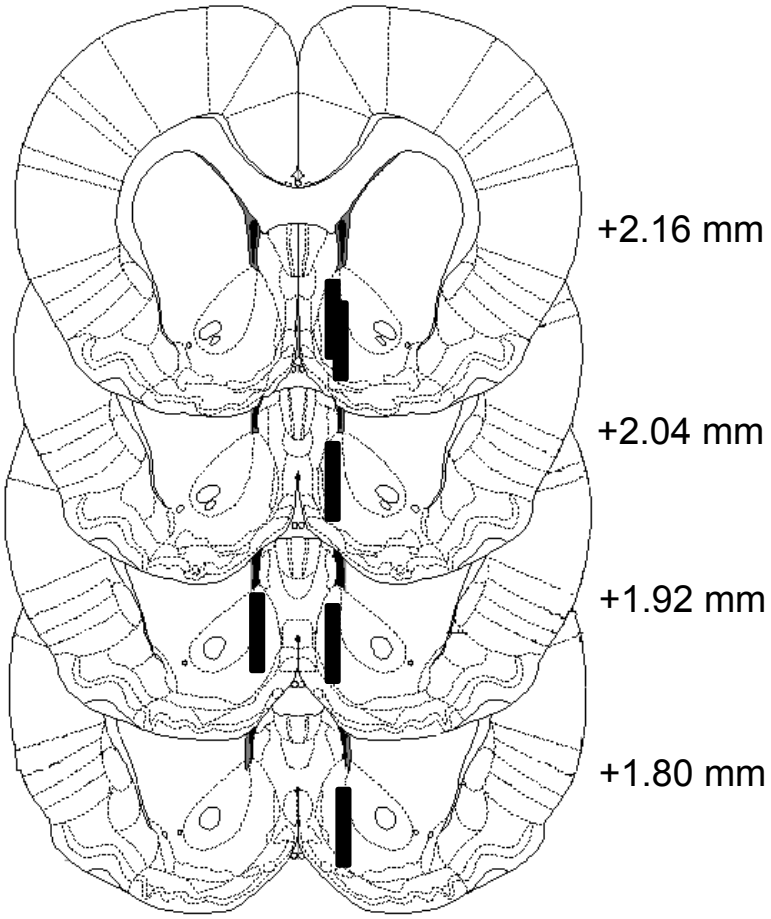

Figure 10 Location of microdialysis probes. Rats were implanted with unilateral guide cannula and microdialysis probes aimed at the nucleus accumbens shell. The semipermeable membrane of the microdialysis probe extended 2mm beyond the guide cannula. Coronal drawings of brain slices are adapted from Paxinos and Watson (2005) atlas.

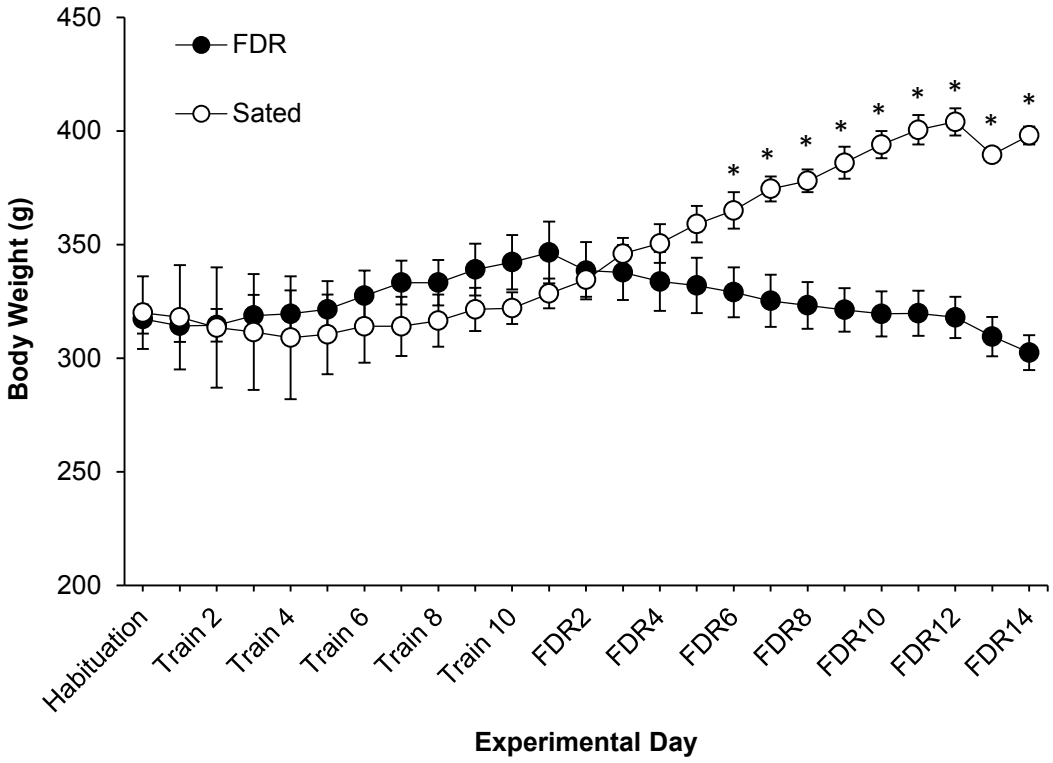

Figure 11 Mean (±SEM) body weights of all rats over the course of the experiment in the food restricted (n = 4) and sated (n = 2) groups. *$p < 0.05$, compared to the sated group.

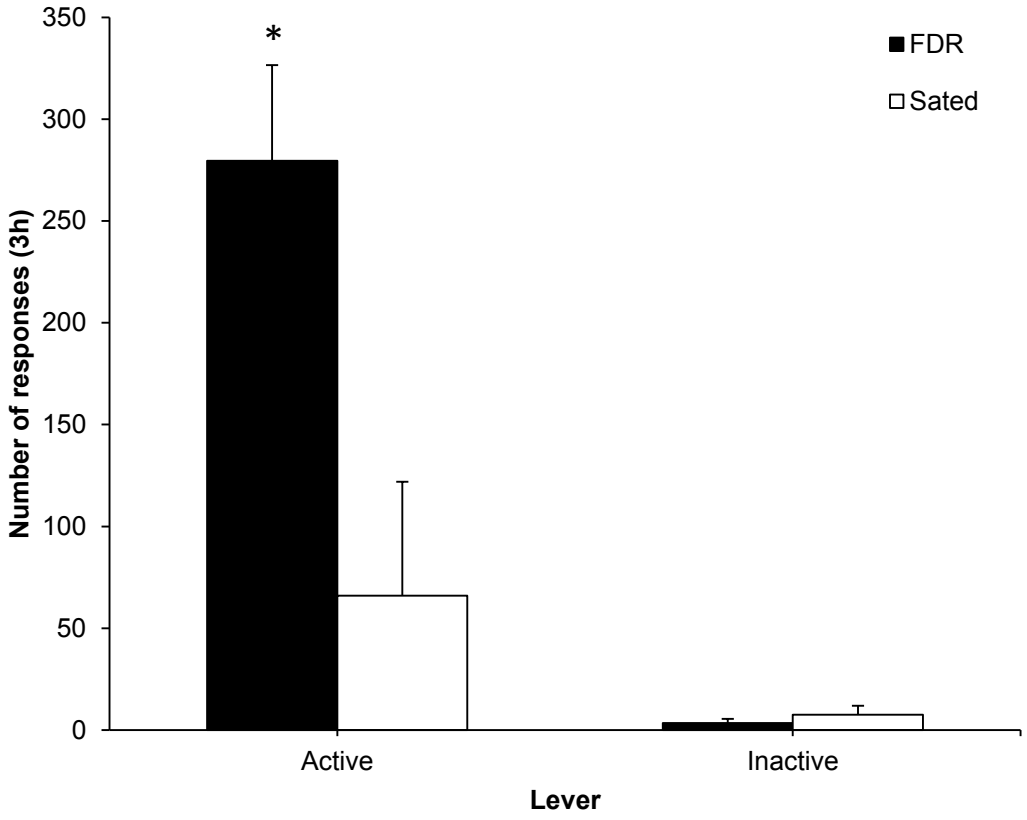

Figure 12 The effect of 14 days of food restriction and abstinence on heroin seeking. Data are mean (+SEM) number of responses made on the active and inactive levers on the test day (food-restriction-day 14) in the food restricted (n = 4) and sated (n = 2) groups. *$p < 0.05$, compared to the sated group.

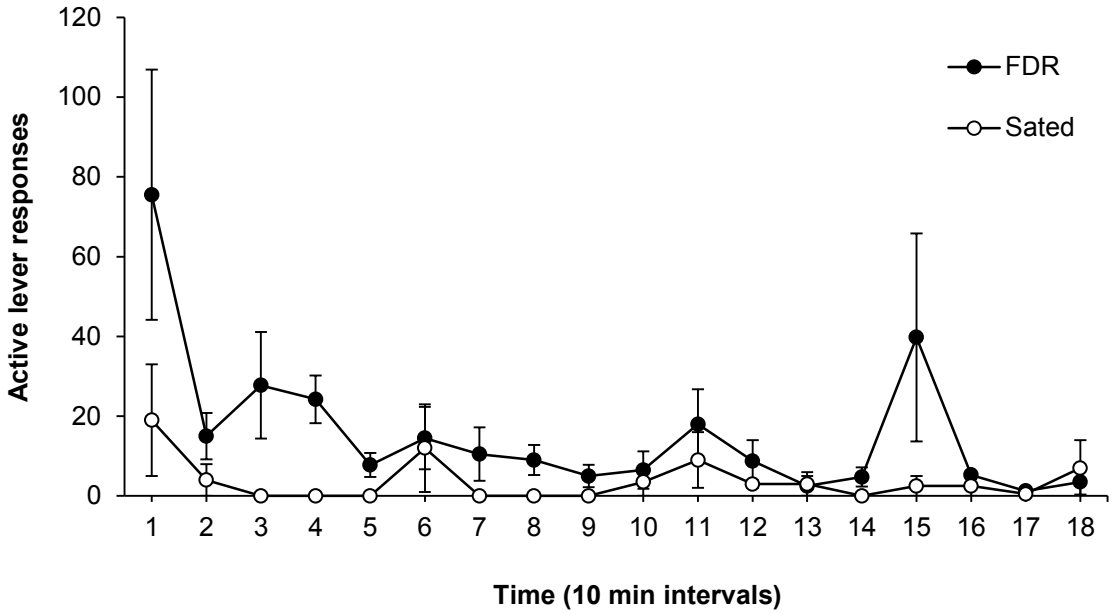

Figure 13 The effect of 14 days of food restriction and abstinence on heroin seeking. Data are mean (+SEM) number of responses made on the active lever in 10 min time intervals during the test for heroin seeking (3 h) in the food restricted (n = 4) and sated (n = 2) groups.

Basal extracellular levels of DA

The mean ± SEM basal DA concentration for the FDR rats was 0.115 ± 0.011 pg/µl and 0.197 ± 0.049 pg/µl for the sated rats. Repeated measures ANOVA revealed significant effects of *time* ($F_{2,8} = 4.657$, $p = 0.046$) and *time* × *food restriction* ($F_{2,8} = 6.062$, $p = 0.025$) and a trend towards statistical significance for the *food restriction* effect ($F_{1,4} = 5.717$, $p = 0.075$). Independent samples t-tests conducted at each of the three time points during baseline revealed no statistically significant differences between the FDR and sated rats during the first and second time point. In contrast, at the third time point FDR rats had a significantly lower concentration of DA compared to the sated rats, $t(4) = 3.742$, $p = 0.02$. The mean ± SEM basal DA concentration at the third time point for the FDR rats was 0.111 ± 0.017 pg/µl and 0.252 ± 0.045 pg/µl for the sated rats.

Effects of chronic food restriction on concentrations of DA during heroin seeking test

Chronic food restriction resulted in increased DA concentrations overall during the 3 h test for heroin seeking (Figure 14). Repeated measures ANOVA revealed a significant effect of *food restriction* ($F_{1,4} = 7.49$, $p = 0.05$), but no effects for *time* and no interaction of *time* × *food restriction*. Regardless of food restriction condition, rats demonstrated a trend for an increase in DA concentrations during the change in context, when they were placed back into the operant conditioning chambers, compared to baseline levels, $t(5) = 2.162$, $p = 0.083$ (Figure 14, "Context change" compared to "baseline" time interval). Only the FDR rats, however, demonstrated a significant increase in DA concentrations compared to baseline levels at the initiation of the drug

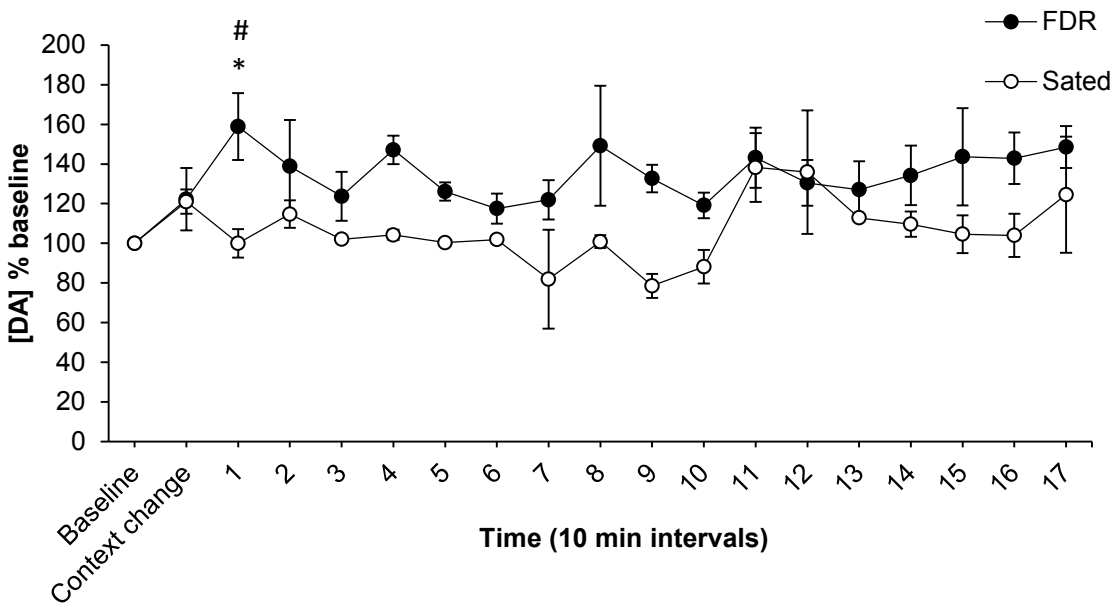

Figure 14 Mean (±SEM) percent change from baseline in extracellular dopamine levels in the nucleus accumbens shell for the food restricted (n = 4) and sated (n = 2) rats in 10 min sample bins during the test for heroin seeking (3 h) on food-restriction-day 14. * significantly different from baseline, $p < 0.05$. # significantly different from sated rats, $p < 0.05$. FDR rats have significantly higher levels of dopamine overall compared to the sated rats during the 3 h test, $p < 0.05$.

seeking test with the resumption of the cues, $t(3) = 3.49$, $p = 0.04$ (Figure 14, time bin "1" compared to "baseline" time interval). The FDR rats also had significantly higher levels of DA concentrations compared to the sated rats during the 10 min interval at the start of the drug seeking test, $t(4) = 3.208$, $p = 0.035$ (Figure 14, time bin "1"). Repeated measures ANOVA revealed no significant effects of *food restriction, time,* or *time × food restriction* for DA's metabolites: DOPAC (Figure 15) and HVA (Figure 16).

Summary

In accordance with our previous findings, food restriction increased heroin seeking following a period of abstinence. As predicted, the FDR rats demonstrated greater responding on the active lever, which was previously paired with heroin, during the drug seeking test compared to the sated rats. Preliminary data suggest that baseline DA levels in the NAc shell may be lower in the FDR rats than in sated rats. Although the overall difference in basal DA levels between the FDR and sated rats was marginal, basal absolute levels of extracellular DA at the third time point in baseline were significantly lower in the FDR compared to the sated rats. Interestingly, re-exposure to the drug context following a period of abstinence increased NAc shell DA levels in both the FDR and sated rats (Figure 14, "Context change" time interval). Furthermore, as predicted, the FDR rats displayed higher levels of extracellular DA in the NAc shell overall during the drug seeking test. Conversely, no differences were found in the extracellular levels of DA's metabolites DOPAC and HVA between groups.

These preliminary data are consistent with reports suggesting that DA in the NAc shell is involved in context-induced reinstatement of heroin seeking (Bossert, et al.,

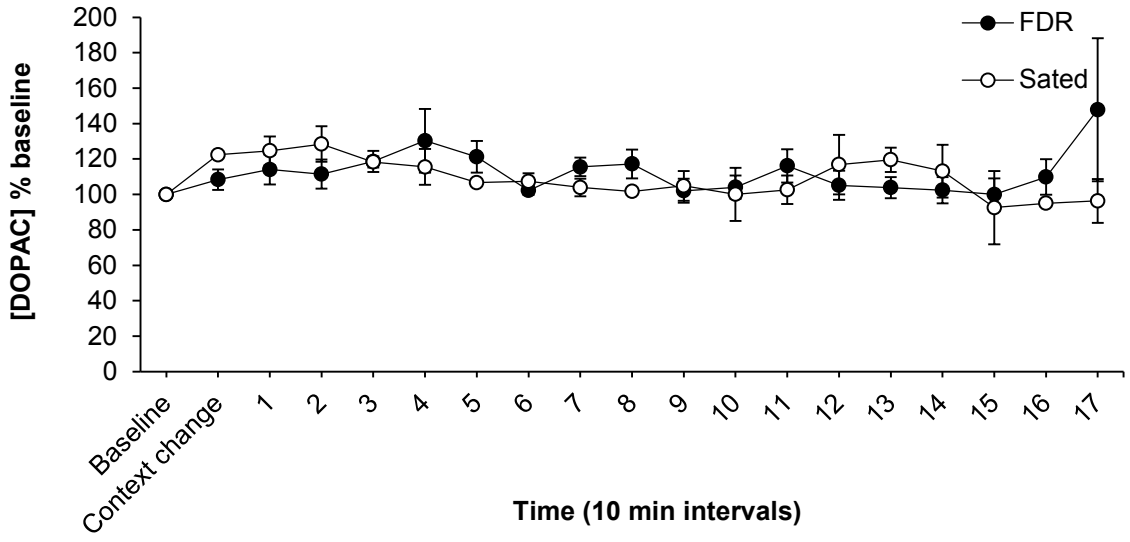

Figure 15 Mean (±SEM) percent change from baseline in extracellular DOPAC levels in the nucleus accumbens shell for the food restricted (n = 4) and sated (n = 2) rats in 10 min sample bins during the test for heroin seeking (3 h) on food-restriction-day 14.

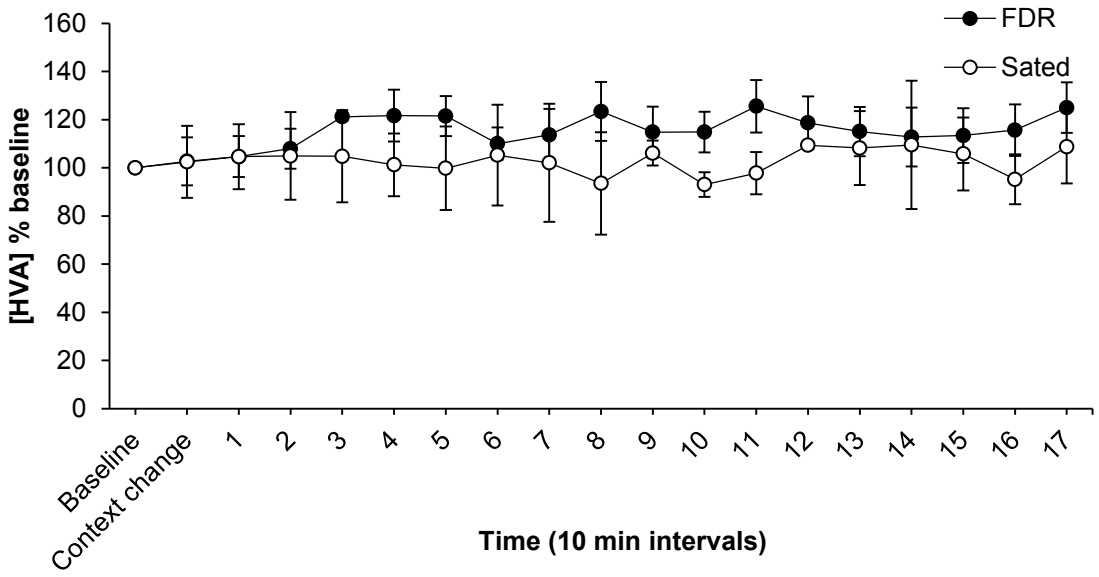

Figure 16 Mean (±SEM) percent change from baseline in extracellular HVA levels in the nucleus accumbens shell for the food restricted (n = 4) and sated (n = 2) rats in 10 min sample bins during the test for heroin seeking (3 h) on food-restriction-day 14.

2007). To summarize, our findings suggest that food restriction-induced changes in DA release in the NAc shell are associated with the augmentation of heroin seeking in food restricted abstinent rats. Discussion of these results and their interpretation will be presented in the general discussion section of this thesis.

General Discussion

The current thesis investigated the effect of chronic food restriction on heroin seeking in abstinent rats. Following a demonstration of a robust increase in heroin seeking in food restricted rats, the neural mechanisms mediating this effect were explored.

As predicted, rats exposed to a 14-day abstinence period demonstrated robust increases in heroin seeking when re-exposed to the drug-associated environment and cues. This effect is in agreement with the findings from previous studies that have shown similar results in both cocaine- and heroin-trained rats (Fuchs, et al., 2006; Neisewander, O'Dell, Tran-Nguyen, Castañeda, & Fuchs, 1996; Shalev, et al., 2000; Shalev, et al., 2001). The most striking outcome of the studies described in Chapter 1, however, was that the chronically food restricted rats displayed dramatically higher levels (>250%) of heroin seeking compared to the sated rats. This augmentation of heroin seeking induced by food restriction was a reliable and robust effect, as demonstrated in both Chapters 1 and 2.

Interestingly, re-feeding the rats for a period of 24 h completely eliminated the effect of food restriction on drug seeking. When the re-feeding period was reduced to 2 h, the augmentation of heroin seeking was attenuated so that there was no longer a statistically significant difference between the FDR and sated rats. Furthermore, when the duration of the food restriction phase was reduced to 3 days at the end of the 14 day abstinence period, there was no increase in heroin seeking observed in the FDR rats compared to the sated rats. Finally, although an acute regimen of food restriction in

Experiment 5 did not augment heroin seeking, re-restricting the previously FDR rats for 5 additional days following the 24 h re-feeding resulted in an increase in heroin seeking compared to the sated rats (Experiment 2). Based on these results, it can be concluded that a concurrent state of hunger and a chronic regimen of food restriction are necessary to augment heroin seeking following a prolonged abstinence period.

Preliminary data presented in Chapter 2 indicate that the food restriction-induced augmentation of heroin seeking may be mediated, at least in part, by DA transmission in the NAc shell. Re-exposure to the drug-associated context following a 14-day period of abstinence resulted in increased NAc shell DA levels in both the FDR and sated groups. There also appeared to be a trend for a reduction in baseline DA levels in the NAc shell in the FDR rats compared to the sated rats. Furthermore, the FDR rats displayed higher levels of DA in the NAc shell overall during the drug seeking test, which was matched with concurrent increases in drug seeking behavior. However, there were no differences observed in the extracellular levels of DA's metabolites, DOPAC, and HVA, in the FDR versus sated rats.

Our current findings are consistent with reports from human studies that describe a positive correlation between the level of food restriction and drug-related behaviors (Cheskin, et al., 2005; Hall, et al., 1992; Krahn, et al., 1992), and with substantial evidence for an augmenting effect of dietary restriction on drug taking and seeking, as well as the reinforcing properties of drugs in animals (Carr, 2007; Carroll & Meisch, 1984; Stuber, et al., 2002). Moreover, the present data extend our previous demonstration of an acute food-deprivation-induced reinstatement of drug seeking (Shalev, et al., 2000). In addition, the current results parallel those that demonstrate reinstatement of

extinguished heroin seeking following 10 days of chronic food restriction (Shalev, 2011). The failure, in the current data, to observe effects of short term food restriction emphasizes the importance of the chronic property of this mild dietary manipulation in augmenting the effects of drug-associated cues on drug seeking. At the present time the reason for the different outcomes of acute versus chronic food restriction are not entirely clear and should be addressed in future studies. Moreover, the revised model of relapse presented in Chapter 1, which contains an abstinence period rather than extinction of drug seeking, may be more clinically relevant since human drug users generally do not experience extinction contingencies while abstinent from drug use.

Dopamine release in the NAc and food restriction-induced augmentation of heroin seeking

Motivation can be defined as the process by which organisms react to stimuli in relation to their predicted outcomes to promote the survival of the organism and the species (Di Chiara, 2002; Dickinson & Balleine, 1994; Toates, 1998). Learning the predictive relationships, or contingencies, between salient stimuli and responses that will lead to beneficial outcomes is a key aspect of motivation (Di Chiara, 2002).

Various researchers have proposed hypotheses that suggest that DA transmission is key in mediating the incentive and motivational properties of salient stimuli. In the 1980's Roy Wise proposed the anhedonia hypothesis, which suggests that DA transmission mediates the impact of the hedonic properties of rewards on behavior (Wise, 1982). He suggested that DA mediates the motivational properties of rewards that are primary unconditioned reinforcers in addition to affecting the motivational properties of

secondary conditioned reinforcers (Wise, 1982). In opposition to Wise's anhedonia hypothesis, Robinson and Berridge have put forth the incentive-sensitization theory (Robinson & Berridge, 1993). This theory proposed that DA systems are critical for the "wanting" or incentive salience of stimuli but not necessarily for the "liking" or the hedonic response elicited by the stimuli (Berridge & Robinson, 1998). Robinson and Berridge's studies suggest that DA transmission in the NAc can magnify the "wanting" of a reward triggered by a reward related cue (Wyvell & Berridge, 2001).

Although there are different neural circuits mediating reward-related behavior for drugs of abuse and natural reinforcers, there is also substantial overlap (Di Chiara, 2005; Kelley & Berridge, 2002). Adaptations in the brain in response to food restriction likely evolved as an adaptive function during times of food scarcity. It is possible that food restriction increases the motivational state of an organism by enhancing the incentive motivational effects of food-related cues (Carr, 1996, 2011). The incentive-motivating effects of external stimuli are dependent on the internal state of the organism (Stewart, de Wit and Eikelboom, 1984), and increasing the rewarding efficacy of food when the organism has an energy deficit is of adaptive value (Bindra, 1978). This enhancement of food reward and the salience of associated cues may transfer over to drugs of abuse, likely because of the shared neural substrate (Di Chiara et al., 1993; Kelley & Berridge, 2002). Since mesolimbic DA is strongly indicated in motivational processes (Wise, 2004), food restriction-induced sensitization of DA transmission in the NAc may increase the incentive motivational effects of the cues related to drug reward, resulting in higher behavioral effectiveness.

One way food restriction could sensitize response to motivationally relevant stimuli is through a reduction in basal DA levels so that the organism is sensitive to increased DA transmission in response to salient stimuli. Evidence supporting this hypothesis finds that a regimen of severe food restriction resulting in 20-30% body weight loss within 7-10 days, decreases basal levels of extracellular DA in FDR rats by approximately 50% compared to sated rats (Pothos, Creese, & Hoebel, 1995). The preliminary findings presented in Chapter 2 also support the aforementioned hypotheses; however, the difference in DA decrease may be accounted for by the fact that our food restriction regimen was not as severe as that used by Pothos and colleagues (1995). It is, therefore, possible that the mild food restriction used here sensitized the DA system in the NAc, as indicted by the lower basal levels of DA in the FDR rats. Furthermore, we find that DA levels in the NAc shell are increased in the FDR rats compared to the sated rats throughout the duration of the drug-seeking test, suggesting a sensitized response in these animals.

In contrast to our findings, and by using a procedure similar to the one in our experiments, Neisewander and colleagues reported that re-exposure to the drug-training context and the discrete cues previously paired with cocaine infusions resulted in an increase in extracellular levels of DA in the amygdala, but not the NAc (Neisewander, et al., 1996; Tran-Nguyen et al., 1998). One possible explanation for these discrepancies is that Neisewander and colleagues did not distinguish between the subregions of the NAc. It is possible that changes in DA release only occur in the shell and not the core in response to re-exposure to drug-associated cues, and consequently, subtle differences in extracellular DA levels might be obscured. Secondly, the present experiment assessed

DA levels following food restriction over a period of abstinence. The sated rats in our experiment demonstrate only a slight trend for an increase in DA when re-exposed to the drug-associated context, which is in accordance with Neisewander et al.'s findings.

Carr and colleagues reported that food restriction can affect DA dynamics. For example, chronic food restriction was shown to decrease the synaptic activity (V_{max}) of the DA transporter (DAT) in the striatum (Patterson et al., 1998), and upregulate striatal cell signaling following activation of post-synaptic DA D1 receptors (Carr, 2007). Thus, the sensitizing effect of food restriction on DA transmission probably involves post-synaptic adaptations, in addition to the pre-synaptic effects on DA release demonstrated here. Post-synaptic adaptations in FDR rats will be investigated in future studies in our laboratory.

The endocrine system and food restriction-induced augmentation of heroin seeking

Over the last decade it has been established that peripheral endocrine adiposity signals that are involved in long-term body weight regulation, e.g. insulin and leptin (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006), and the orexigenic gastrointestinal peptide ghrelin can directly interact with midbrain reward systems, and modulate the hedonic and rewarding properties of natural rewards as well as drugs (Cummings, Naleid, & Figlewicz, 2007; Figlewicz, 2003). For example, chronic leptin administration reversed food-restriction-induced CPP to a small amount of sucrose (Figlewicz, Higgins, Ng-Evans, & Havel, 2001) and acute intracerebroventricular (i.c.v.) leptin infusion attenuated the effectiveness of LHSS in food restricted rats (Fulton, et al., 2000). In addition, insulin (i.c.v.) reversed the threshold lowering effect of food

restriction in the LHSS procedure (Carr, Kim, & Cabeza de Vaca, 2000). Moreover, acute leptin administration (i.c.v.) was shown to block food-deprivation-induced reinstatement of extinguished heroin seeking (Shalev, et al., 2001). Interestingly, central leptin administration did not alter the rewarding effects of amphetamine in FDR rats, as assessed with LHSS paradigm (Hao, Cabeza de Vaca, Pan, & Carr, 2006), suggesting that food-restriction-induced hypoleptinemia is not critically involved in the enhancement of drug reward in restricted animals. Moreover, the attenuation of the food restriction effect following an acute (2 h) re-feeding period (Experiment 3), which would have minimal impact on circulating levels of leptin (Schneider, Blum, & Wade, 2000) suggests that leptin has no or only a minor role in food restriction induced augmentation of heroin seeking in abstinent rats. Future studies will clarify leptin's role in the augmentation of heroin seeking by food restriction by assessing plasma leptin levels following chronic food restriction and re-feeding, and, if changes are observed, by chronic and acute manipulations of leptin levels before the drug-seeking test.

Recent studies suggest that ghrelin plays an important role in reward processes induced by natural stimuli, as well as by drugs such as cocaine, amphetamine, alcohol, and nicotine. For example, ghrelin receptor antagonists attenuated cocaine, nicotine, and amphetamine-induced CPP, suppressed alcohol intake and abolished alcohol-induced CPP (Jerlhag, Egecioglu, Dickson, & Engel, 2010; Jerlhag et al., 2009; Jerlhag & Engel, 2011). In contrast, our laboratory has recently reported that although central ghrelin administration increased breakpoint on a progressive ratio schedule of heroin reinforcement, treatment with a ghrelin receptor antagonist had no effect on acute food-deprivation-induced reinstatement of heroin seeking (Maric, et al., 2011). In humans,

ghrelin infusions resulted in an increased neural response to food pictures in areas of the brain associated with reward processing (Malik, McGlone, Bedrossian, & Dagher, 2008). Importantly plasma levels of ghrelin increase during periods of food restriction, and drop sharply following a meal (Drazen, Vahl, D'Alessio, Seeley, & Woods, 2006; Tschop, Smiley, & Heiman, 2000), a pattern that parallels our behavioral findings with food restriction and acute re-feeding. Ghrelin might, therefore, mediate the effect of food restriction on heroin seeking following prolonged abstinence. Additionally, ghrelin receptors are also expressed in the mesolimbic DA circuit (Abizaid et al., 2006), and have been found on midbrain dopaminergic neurons in the VTA (Zigman, Jones, Lee, Saper, & Elmquist, 2006). Moreover, increases in basal plasma ghrelin levels are accompanied by significant increases in extracellular dopamine in the NAc shell but not the core (Quarta et al., 2009). The increase in extracellular NAc shell DA in our food restricted rats, and the reversal of the augmentation of drug seeking following re-feeding, may consequently be mediated by changes in ghrelin levels, since ghrelin levels drop rapidly following the consumption of a meal (Drazen et al., 2006; Tschop, Smiley & Heiman, 2000).

Stress-response pathways and food restriction-induced augmentation of heroin seeking

Prolonged food restriction is considered a stressor, and as such activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated blood plasma levels of the adrenocorticoid, corticosterone (Carr, 1996). Corticosterone levels have previously been correlated with the propensity to self-administer amphetamine and cocaine, and corticosterone is necessary for the food-deprivation-induced sensitization of the locomotor response to psychostimulant drugs and morphine (Piazza & Le Moal, 1996). It

has been previously suggested that exposure to drug-associated cues produces incentive motivational state that then drive drug-seeking (Stewart, de Wit, & Eikelboom, 1984). Food-restriction-induced increase in corticosterone levels may interact with the effects of drug-associated cues to provoke powerful incentive motivational effect. Although this is a compelling mechanism, more recent studies indicate a more limited role for stress-induced corticosterone release in food-restriction-induced sensitization of the rewarding properties of psychostimulant drugs (Carr, 2002), as well as in the acute food-deprivation-induced reinstatement of heroin and cocaine seeking (Shalev, Finnie, Quinn, Tobin, & Wahi, 2006; Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003). Basal levels of corticosterone seem to play a permissive role in food deprivation-induced reinstatement of cocaine seeking. Thus, removal of endogenous corticosterone, via adrenalectomy, attenuated food deprivation-induced reinstatement of cocaine seeking, and this effect was reversed when basal levels of corticosterone were replaced (Shalev, et al., 2003). Additionally, adrenalectomy had no effect on food deprivation-induced reinstatement of extinguished heroin seeking (Shalev, et al., 2006). In contrast, extrahypothalamic corticotropin releasing factor (CRF), a neuropeptide that mediates many of the behavioral and physiological responses to stress (Johnson, Kamilaris, Chrousos, & Gold, 1992), is critically involved in acute food-deprivation-induced reinstatement of extinguished heroin and cocaine seeking (Shalev, et al., 2006). Interestingly, it has been suggested that activation of CRF systems in the NAc shell can enhance the incentive salience (or "wanting") that is assigned to reward cues, thus providing a link between stress-induced pursuit of rewards and reward-associated cues (Pecina, Schulkin, & Berridge, 2006).

A role for the stress component of food restriction in inducing drug seeking behavior is indicated by the finding that heroin seeking was attenuated following both brief (2 h) and prolonged (24 h) undisturbed access to food. These results are in agreement with the previously reported rapid decline in cocaine reinforced behavior in FDR rats following a return to free feeding (Papasava & Singer, 1985). It is important to note, however, that the state of hunger itself is not sufficient to induce an increase in heroin seeking in abstinent rats. Specifically, neither 5 days (Experiment 4) nor 3 days (Experiment 5) of food restriction resulted in increased heroin seeking compared to sated rats. In contrast, a short (5 days) food restriction treatment, following re-feeding in previously food restricted rats (Experiment 2), resulted in pronounced augmentation of heroin seeking. The lack of an effect for short food restriction in Experiment 5 strongly suggests that the difference in effectiveness of short food restriction is not simply a reflection of the repeated tests procedure used in Experiment 4. Rather, these findings suggest a complex interaction between adaptations that occur during the prolonged food restriction period and state of hunger during the drug-seeking test. The adaptations implied above could be in the peripheral energy balance-related signal systems, as well as in the brain pathways involved in the processing of drug and natural rewards.

The role of stress-response pathways, and more specifically, activation of the CRF system and the HPA axis, in the augmentation of heroin seeking by chronic food restriction is currently under investigation in our laboratory.

Methodological considerations

There are two major methodological points worth noting. First, the food restriction regimen we have utilized here is mild compared to most other studies cited. On the test day, the body weights of the FDR rats were 72-80% of the sated rats, or about 90% of their pre-restriction body weight. In contrast, in Carr's or Fulton et al.'s studies (Carr, 2007; Fulton, et al., 2000) rats were food restricted to 75-80% of their pre-restriction body weight. It is unusual to observe a weight loss of 20-25% in healthy humans. This further emphasizes the clinical relevance of the procedure used here. Second, in most of the previous studies that explored the effect of dietary manipulations on drug-seeking behavior, the drug choice was a psychostimulant, while rats in the current series of experiments were trained with heroin. Drug associated behaviors, as well as brain adaptations that might underlie these behaviors, were shown to differ in animals exposed to psychostimulant and opiate drugs (Badiani, et al., 2011). Therefore, at this point it remains unclear how well the previously suggested neuronal mechanisms can explain the behaviors described here. Finally, although preliminary evidence suggests changes in the dopaminergic system of the FDR rats, the sample size in this experiment is very small and the effect should be replicated in order to verify the results.

Conclusion

In summary, the present findings suggest that a mild chronic food restriction regimen during a 14 day period of abstinence will augment heroin seeking as compared to sated rats. Interestingly, re-feeding the previously FDR rats attenuates this effect, suggesting this effect is very sensitive to the feeding state. However, a short period of food restriction is not sufficient to induce an increase in heroin seeking. Therefore, a combination of a chronic regimen of food restriction and a concurrent state of hunger are

necessary to see an augmentation of heroin seeking induced by food restriction. Preliminary findings suggest that food restriction may sensitize the mesolimbic DA circuit so that an increase in DA during re-exposure to the drug-associated context and cues may be mediating the behavioral increase in drug seeking. The use of abstinence in this model of relapse may also be more ecologically valid compared to the reinstatement procedure which includes a period of extinction. Future studies will investigate the neural mechanisms that mediate this food restriction effect by replicating the experiment in Chapter 2, and exploring the involvement of feeding related mechanisms.

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