A Hormonal, Neuronal, And Behavioural Approach To The Augmentation Of Heroin Seeking In Chronically Food-Restricted Rats Under Withdrawal

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ABSTRACT

A hormonal, neuronal, and behavioural approach to the augmentation of heroin seeking in chronically food-restricted rats under withdrawal

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Why can't we stop? Despite one's greatest efforts to remain abstinent, re-exposure to drug-associated cues can elicit drug-craving and promote relapse after withdrawal. Interestingly, hunger can increase the vulnerability to relapse. In chronically food-restricted, compared to sated rats, we demonstrated augmented heroin seeking under withdrawal. However, the mechanisms responsible for this susceptibility remain unclear. Here, we evaluated the hormonal, neuronal, and behavioural processes that may contribute to the enhanced vulnerability to drug seeking in calorically-restricted rats.

Estradiol (E2) may facilitate, while progesterone may attenuate drug seeking; however, we are the first to assess these hormones' role in calorically-restricted, heroin-seeking rats. Removal of the ovaries and subsequent progesterone replacement yielded no effects. However, E2 replacement attenuated the augmented heroin seeking observed in food-restricted rats, providing a therapeutic target for drug-addiction treatment in females.

Next, we investigated the role of the glutamate GluA1 and phosphorylated GluA1 (p-Ser845-GluA1) subunits, which contribute to the *long-term* relapse vulnerability of abused drugs. Re-exposure to heroin cues, compared to heroin-trained rats not re-exposed to the cues, increased p-Ser845-GluA1 protein levels in the nucleus accumbens (NAc) shell. Reduced NAc shell GluA1 and p-Ser845-GluA1 protein levels were also observed in heroin-trained compared to drug-naïve rats. No changes were observed due to dietary restriction. As we did not dissociate membrane-bound and cytoplasmic receptors, our findings may be a limitation of the

procedure employed. Further work is needed to clarify the role of the GluA1 receptor in chronically food-restricted, heroin-seeking rats.

Lastly, we investigated whether augmented heroin seeking in our food-restricted rats was a function of the cues' association with the drug, or the inherent value of light cues alone. While some light-cue-specific effects were observed, we concluded that caloric restriction contributes to the motivational properties of light cues as a result of their heroin association.

Collectively, we demonstrate the value of a multi-dimensional approach to the understanding a novel behavioural procedure. While we have answered some questions on the hormonal, neuronal, and behavioural mechanisms underlying the enhanced vulnerability to drug abuse in calorically-restricted rats, we have importantly presented many questions to drive future research.

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CONTRIBUTION OF AUTHORS

Chapter 2

Firas Sedki: Contributed in the experimental designs, conduct of the behavioural experiments, hormone administration, blood collection, and plasma estradiol/progesterone determination and surgical procedures. I have also contributed to the analysis strategy, the final data analysis and writing of all versions of the manuscript.

James Gardner Gregory: Contributed to the analysis strategy, conduct of the behavioural experiments, administration of study hormones, blood collection, and surgical procedures for Experiment 1. James was also involved in strategizing future experiments.

Adriana Luminare: Contributed to the conduct of the behavioural experiments and hormone administration for Experiments 2 and 3.

Tracey M. D'Cunha: Contributed to surgical procedures and blood collection across all experiments, as well as the reviews of the manuscript revisions.

Uri Shalev: Contributed to the experimental designs, analysis strategy, and to writing all versions of the manuscript.

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Chapter 3

Firas Sedki: Contributed in the experimental designs, conduct of the behavioural experiments, blood and tissue collection, tissue preparation, conduct of the Western Blots

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Staci Angelis: Contributed to the conduct of the behavioural experiments, tissue collection, tissue preparation, and conduct of the Western Blots.

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Appendix

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LIST OF ABBREVIATIONS

ACF	Animal care facility
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA receptor
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BSR	Brain stimulation reward
BW	Body weight
CH	Cholesterol
CI	Confidence interval
CIHR	Canadian Institute of Health and Research
Cp	Caudate putamen
CPP	Conditioned place preference
CR	Conditioned response
CRF	Corticotropin releasing factor
CS	Conditioned stimulus
D-R	Dopamine receptor
DA	Dopamine
dmPFC	Dorsomedial prefrontal cortex
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
E2	Estradiol
EB	17β-estradiol 3-benzoate
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FDR	Food restriction
FI	Fixed interval
FR	Fixed ratio
GHSR	Growth hormone secretagogue receptor

GluA-R	Glutamate receptor
H.A.L.T	Hungry. Angry. Lonely. Tired
HPA	Hypothalamic-pituitary-adrenal
i.v.	Intravenous
ICSS	Intracranial self-stimulation
iGlur	Ionotropic glutamate receptor
IL	Infralimbic cortex
ITI	Inter trial interval
LRb	Leptin receptor-b
LTD	Long-term depression
LTP	Long-term potentiation
mGlur	Metabotropic glutamate receptor
mPFC	Medial prefrontal cortex
MSN	Medium spiny neuron
n	Sample
NAc	Nucleus accumbens
NAM	Negative allosteric modulator
NIH	National Institute of Health
NMDA	N-methyl-D-aspartate
OIL	Sesame oil
OVX	ovariectomy
P	Progesterone
PAM	Positive allosteric modulator
PFC	Prefrontal cortex
PIT	Pavlovian-instrumental transfer
PL	Prelimbic cortex
PR	Progressive ratio
Ser	Serine

UR	Unconditioned response
vmPFC	Ventromedial prefrontal cortex
VTA	Ventral tegmental area
W	Withdrawal

Chapter 1 - GENERAL INTRODUCTION

"These people are not victims, they chose to do drugs, deal with what happens. Why would parents of a 2 year old decide it would be smart to do street drugs anyways?"
- Anonymous comment to "Fentanyl deaths are a Canada-wide 'disaster'" by Aleksandra Sagan, CBC News

1.1 - Seventy-eight.

A requirement for completing a dissertation consists, among others, of complaining about the hardships of completing a dissertation. We routinely find ourselves in existential thought, contemplating the merits, worth, and purpose of what we do. Not long ago, as I sat in contemplation, the importance of our field of study dawned on me. *Seventy-eight.* Today, in North-America alone, over seventy-eight individuals lost their lives as a result of an opiate related drug overdose (Rudd, Aleshire, Zibbell, & Gladden, 2016). This alarming statistic is a grim reflection of the 42,249 people that die each year from prescription and non-prescription opiate related overdoses, accounting for 66.4% of all 63,632 drug overdose deaths reported in the United States in 2016 (Seth, Rudd, Noonan, & Haegerich, 2018). The disturbing nature of these numbers are further capitulated by the fact that deaths from drug overdoses are over 20% greater than deaths resulting from gun violence or motor vehicle accidents (Seth, et al., 2018).

North America is currently in the midst of an opiate epidemic, prompted not only by recreational and non-therapeutic drug use, but largely by the high onset of prescribed opiates. While typically prescribed for pain management, it is the inability to abstain from opiate use following the cessation of pain that has become a worldwide concern. In fact, from 2004 to 2011, emergency department visits resulting from the misuse of prescription opiates have risen 153% (Substance Abuse and Mental Health Services Administration, 2013), and over 1000 people are treated daily in emergency departments for the misuse of prescription opiates

(Substance Abuse and Mental Health Services Administration, 2013). Furthermore, admissions to drug treatment programs and the rates of death due to prescription opiate misuse have nearly quadrupled in the last decade (Substance Abuse and Mental Health Services Administration, 2014). Today, over 10,000,000 individuals are estimated to use prescription opiates non-medically (Tetrault & Butner, 2015). More unsettling is the notion that an acquired dependency to the non-medicinal use of prescription opiates can also precede the transition to maladaptive heroin use in humans (Kandel & Kandel, 2015).

1.2 - On thin ice.

From a clinical perspective, a dependent drug user is one that persists in, increases, and returns to drug use following periods of abstinence, despite any negative consequences (American Psychological Association, 2013; see Table 1.1 for a comprehensive list of clinical criteria). These persistent drug cravings impose a life reminiscent of walking on thin ice where at any moment, even after a long uneventful period, an unexpected crack can drown any hope of remaining abstinent (Robinson & Berridge, 1993; Wise & Bozarth, 1987). Indeed, often reported is an incubation period whereby abstinent drug users report increases in drug craving over time (Gawin & Kleber, 1986), contrary to the expectation that such feelings would diminish.

The approximate $200 billion in annual costs related to crime, loss of productivity, and health care, exacerbated by the perpetual nature of drug addiction, indicate that an adequate treatment for, or a clear understanding of, the underlying mechanisms that drive drug abuse and relapse remain elusive (U.S. Department of Justice National Drug Intelligence Center, 2011). Currently, one of the main forms of treatment for drug relapse involves acute, rescue pharmacological intervention, which reflects a poor model of chronic disease management (White, Boyle, & Loveland, 2002). As a result, treatment for drug addiction remains widely ineffective, with as many as 80% of abstinent drugs users returning to active substance use within one year following treatment (Hser, Hoffman, Grella, & Anglin, 2001). Despite the

persistent and recurrent negative health-related, social, and psychological consequences, the irrational need to seek and take drugs in addicted individuals remains powerful. Thus, when

Diagnostic Criteria for Opioid Use Disorder	
A problematic pattern of opioid use leading to clinically significant impairment or distress, as manifested by at least two of the following, occurring within a 12-month period*:	
1	Opioids are often taken in larger amounts or over a longer period than was intended.
2	There is a persistent desire or unsuccessful efforts to cut down or control opioid use.
3	A great deal of time is spent in activities necessary to obtain the opioid, use the opioid, or recover from its effects.
4	Craving, or a strong desire or urge to use opioids.
5	Recurrent opioid use resulting in a failure to fulfill major role obligations at work, school, or home.
6	Continued opioid use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of opioids.
7	Important social, occupational, or recreational activities are given up or reduced because of opioid use.
8	Recurrent opioid use in situations in which it is physically hazardous.
9	Continued opioid use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
10	Tolerance, as defined by either of the following:
a	A need for markedly increased amounts of opioids to achieve intoxication or desired effect.
b	A markedly diminished effect with continued use of the same amount of an opioid.
11	Withdrawal, as manifested by either of the following:
a	The characteristic opioid withdrawal syndrome
b	Opioids (or a closely related substance) are taken to relieve or avoid withdrawal symptoms.
***Severity is determined according to the number of criteria met.**	

Table 1.1. List of the Diagnostic criteria for opioid use disorder, as per the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition.

one asks, *"Why would parents of a 2 year old decide it would be smart to do street drugs anyways?"*, the simplest answer is, we do not know. The complex nature of drug relapse most likely involves disturbances in hormonal, neuronal, and behavioural elements that underlie drug relapse. Accordingly, in the current thesis, hormonal, neuronal, and behavioural approaches were employed to progress the development, and understanding of a model of drug relapse developed in our laboratory.

1.3 - *The difficulty in studying relapse in humans.*

Controlled laboratory experiments suggest that the initiation of drug use is often provoked upon exposure to stressful life events, re-exposure to the formerly abused drug, or re-exposure to cues previously associated with the drug (Childress et al., 1993; Sinha, 2001). However, two of the major limitations to the study of relapse in humans is the retrospective and correlative nature of the reports. Studies applying retrospective data collection are inherently limited by the inconsistent ability of a user to correctly recall the motivation for their drug lapse (McKay, Franklin, Patapis, & Lynch, 2006). To address these limitations, some studies (Epstein et al., 2009; Fatseas et al., 2011; Preston et al., 2018) employed a set of electronic devices, allowing for real time data collection concerning the degree of drug craving, and moments preceding drug relapse. Indeed, these data reveal that the provocation of drug use can follow exposure to stress, drugs and drug-associated cues. Given the demonstrated impact of drug-associated cues on drug-taking and -seeking behaviours, as described below, we have chosen to focus on the hormonal, neuronal, and behavioural mechanisms that underlie the effects of drug-associated cues. To fully comprehend the rationale for this focus, we first present evidence on the significance of drug cues in human drug users.

1.4 - *Drug-associated cues and their impact on relapse.*

Exposure to drug-associated cues can drive drug craving and increase the propensity for drug lapses in human users. This is consistent with the considerable evidence that exposure to drug

cues in laboratory settings increases an individual's subjective craving for the drug (Carter & Tiffany, 1999). Indeed, a survey of 2431 smokers reported exposure to smoking-related cues as one of the primary antecedents to relapse (Zhou et al., 2009). Recently, this evidence was corroborated by real time data collected using mobile electronic devices, where encounters with drug cues repeatedly preceded strong elevations in subjective craving (Fatseas et al., 2015). In addition, a strong association between drug cues and subsequent substance use was revealed, and suggested to be largely mediated by craving intensity (Fatseas et al., 2015).

The influence of drug cues has also been investigated with respect to the affective states they may induce in drug users. To test the emotional impact of images, researchers often employ an acoustic startle response procedure, where one can measure a behavioural response evoked by a sudden and intense auditory stimulus. Here, a strong startle response has been associated with a general increase in anxiety, which is further potentiated by the presentation of unpleasant images, and inhibited by the presentation of pleasant images (Bradley, Cuthbert, & Lang, 1993). With respect to abused drugs, it was demonstrated that presenting alcohol or smoking cues to users inhibited the startle response, indicating the potential appetitive nature of these cues (Geier, Mucha, & Pauli, 2000; Mucha, Geier, Stuhlinger, & Mundle, 2000). However, conclusions from these data should be interpreted with caution, as others have reported that the inhibition of the startle response in smokers is not specific to images of smoking cues alone (Orain-Pelissolo, Grillon, Perez-Diaz, & Jouvent, 2004), and may also be observed in response to neutral cues.

Another attempt to overcome the correlational nature of retrospective studies includes the development of a two-sound differential conditioning procedure in smokers. Here, one auditory cue is paired with smoking, while a second, control cue is presented in a non-paired manner. Smokers have shown enhanced preferences for smoking-paired cues, and were found to draw more on their cigarette in the presence of the smoking cue compared to the control cue (Mucha, Pauli, & Angrilli, 1998). These data indicate the importance of drug cues not only in promoting relapse, but in increasing drug use as well. Interestingly, the acute effects of

smoking, (e.g., increased heart rate and skin conductance) were not affected by smoking cues (Mucha et al., 1998). This suggests that relapse may not result from the alleviation of physical distress, but rather from other underlying, and potentially motivating, properties of the drug cues themselves.

Evidence of the influential nature of drug-associated cues has also been gathered using the conditioned place preference (CPP) procedure, where subjects develop a preference for an environment where a drug is experienced. In humans, participants report an augmented preference for a *d*-amphetamine-associated room compared to a control room (Childs & de Wit, 2009). A follow-up study examining the effects of repeated *d*-amphetamine treatments has indicated a sensitization, or increase, of the craving response to the drug environment following multiple injections (Childs & de Wit, 2013). Interestingly, this preference for drug-associated contextual cues was not correlated with the subjective effects (i.e., 'liking') of the drug (Mayo et al., 2013), suggesting that cue-conditioning is more complex than a simple association with the euphoric effects of drugs. As such, these data act only as rudiments to a causative link between cue exposure and drug relapse in humans.

The identification of factors which modulate how cues are perceived can also impact the development of treatments aimed to alleviate the intense craving resulting from cue exposures. While users are exposed to a wealth of external factors, here we focus on how dietary manipulations impact relapse in humans, and drug-seeking behaviours in rodents.

1.5 - *The issue of hunger in relapse.*

Individuals who have joined Narcotics Anonymous programs are often told to satisfy a set of basic tenets, termed H.A.L.T. (Hungry. Angry. Lonely. Tired), to suppress the desire and need for drug use. If these conditions are met, it is postulated that the ability to maintain an abstinent lifestyle increases. It is therefore implied that feelings of hunger, the first tenet, must be treated to reduce the risk of relapse. In accordance, anecdotal evidence suggests that malnourishment during times of war has been linked to increases in nicotine and caffeine consumption (Franklin,

Schiele, Brozek, & Keys, 1948), and that malnourished Peruvian Indians were also reported to chew more coca leaves (Hanna & Hornick, 1977). In addition, clinical studies have shown that a high vulnerability for use and relapse to drugs is present in dieters and individuals with low body mass index (Hall, Tunstall, Vila, & Duffy, 1992; Rosse, Deutsch, & Chilton, 2005). Furthermore, patients subjected to gastric bypass surgery have been found to exhibit an increased likelihood in developing alcoholism and drug addiction (Conason et al., 2013; Ivezaj, Saules, & Wiedemann, 2012). Lastly, that a positive correlation exists between the severity of diet and the risk of drug taking in young women (Krahn, Kurth, Demitrack, & Drewnowski, 1992), that up to one third of cocaine users meet the diagnostic criteria for an eating disorder (Jonas, Gold, Sweeney, & Pottash, 1987), as well as recent evidence for increased cigarette use among calorically restricted abstinent smokers (Cheskin, Hess, Henningfield, & Gorelick, 2005), collectively suggests a strong link between dietary restriction and drug intake. Therefore, due to the considerable impact on human drug users (Franklin et al., 1948; Krahn et al., 1992) and the high comorbidity with eating-related disorders (Holderness & Brooks-Gunn, 1994), dietary manipulations are highly relevant to the study of relapse.

While reports on drug abuse in humans and its impact on the progress of our understanding is invaluable, such reports are often ethically limited and mostly correlational in nature. Moreover, the neurobiological correlates of drug abuse in humans are often discussed with data that are limited in spatial and temporal resolution, requiring the support of complementary causative data that can be collected using animal models of relapse.

1.6 - Developing animal models of relapse.

The translational validity of animal models of relapse forms a critical component in the study of underlying behavioural and neural mechanisms of relapse. A strong argument for the use of animal models of relapse resides within evidence that the same clinical criteria which define drug addiction in humans are also exhibited in rodents. This includes the inability to cease or limit drug intake, the preoccupation with, and increased motivation to obtain drugs, and the

continued use despite harmful consequences (American Psychological Association, 2013; Deroche-Gamonet, 2004; Vanderschuren & Everitt, 2004). This homogeneity across species is further supported by the discovery that addiction-like behaviours in animal models are observed only in a minority of rodents. In those exposed to long-term drug self-administration procedures, these addiction-like behaviours do not decline in the presence of aversive stimuli, and that they do not extend to non-drug rewards (Deroche-Gamonet, 2004; Vanderschuren & Everitt, 2004). Given that stress (Shaham & Stewart, 1995; Shaham, Rajabi, & Stewart, 1996), re-exposure to an abused drug (de Wit & Stewart, 1981a), and re-exposure to drug associated cues (de Wit & Stewart, 1981a) can promote the return to drug seeking in animal models of relapse, which strongly suggests important similarities in behaviour across species.

The most commonly used animal model of relapse which also serves as the foundation for the procedure used in our laboratory, is the self-administration-based reinstatement procedure. Briefly, animals are trained to perform an operant response, often paired with the presentation of discrete cues (e.g., a light), to acquire a drug. This behaviour is then extinguished by keeping all training conditions similar, with the exception of drug availability. Rodents can then be exposed to any number of conditions thought to initiate craving and relapse in humans, such as exposure to drug-associated cues (de Wit & Stewart, 1981b; Shaham, Shalev, Lu, de Wit, & Stewart, 2003). Indeed, reintroduction to previously drug-paired environments or to discrete drug-associated cues have been shown to successfully reinstate extinguished drug seeking (Shalev, Grimm, & Shaham, 2002).

The intuitive appeal of the reinstatement model is derived from the fact that the main factors known to trigger relapse in humans also induce reinstatement of drug seeking in laboratory animals (Epstein, Preston, Stewart, & Shaham, 2006). Nevertheless, human addicts rarely undergo explicit periods of extinction (Fuchs, Lasseter, Ramirez, & Xie, 2008; Katz & Higgins, 2003), and instead experience short or prolonged periods of withdrawal preceding the resumption of drug seeking. These behaviours are also distinguished by important differences in the neural circuitry involved in abstinence versus extinction learning (Fuchs et al., 2008;

Fuchs, Branham, & See, 2006). The inactivation of the caudate putamen (Cp), for example, attenuates cocaine seeking following withdrawal. However, the inactivation of other structures implicated in the reinstatement of extinguished drug seeking was without effect (Fuchs et al., 2006). Furthermore, the investigation of chronic relapse triggers on drug seeking cannot be accomplished without a significant impact to the extinction process, or, if applied post-extinction, on the effects of spontaneous recovery.

We have therefore adapted a more clinically relevant model, where chronically food-restricted rats with a history of heroin self-administration are tested following a prolonged withdrawal period. This novel procedure constitutes three phases: animals are first trained to self-administer a drug paired with auditory and visual stimuli (i.e., self-administration phase). Then, rats are moved to a different context and allowed unrestricted or restricted access to chow (i.e., withdrawal phase). Finally, rats are returned to the drug-taking environment for a drug-seeking test under extinction conditions (i.e., test phase). Recently, our laboratory has reported a robust (>250%) food-restriction-induced augmentation in heroin seeking, when compared to sated controls (D'Cunha et al., 2013). The development of a novel procedure requires a multidimensional foundation to not only elucidate the underlying mechanisms that mediate any observed behaviour, but to also guide the direction of future investigations targeted at their understanding. To that end, we have addressed the hormonal, neuronal, and behavioural mechanisms that may contribute to the augmentation of drug seeking following exposure to a mild caloric restriction regimen.

1.7 - The hormonal approach: Understanding the role of ovarian hormones in food-restriction-induced drug-cue seeking

A thorough understanding of the behavioural and neurobiological underpinnings of drug relapse requires the study of males and females. Identifying differences and similarities across biological sexes contributes not only to our grasp of these behavioural and biological processes but is also fundamental to the development of more targeted treatments across

populations. We therefore present a set of experiments (Chapter 2) with the goal of elucidating whether chronically food-restricted female rats will augment heroin-seeking behaviour and the involvement of sex hormones.

1.7.1 - Finding sex differences in drug addiction.

Despite higher rates of drug use among men, recent epidemiological studies have suggested that the gap in drug use between men and women has narrowed. In recent surveys on alcohol dependence, a 3:1 male to female ratio has been observed, in comparison to the previously reported 5:1 ratio endorsed in the 1980s (Hasin, Stinson, Ogburn, & Grant, 2007; Robins & Regier, 1991). The sizeable gaps are now believed to have resulted from poor access, rather than the vulnerability to drug use (Van Etten & Anthony, 2001; Van Etten, Neumark, & Anthony, 1999).

The most alarming disparities among sexes, however, continues to be the accelerated escalation from substance use to dependence, as well as the faster entry into treatment programs characteristic of women compared to men (Becker & Hu, 2008; Becker & Koob, 2016; Lynch, Roth, & Carroll, 2002). This accelerated progression, termed telescoping, has been well documented across a variety of drug classes including alcohol, stimulants, cannabis, and opiates (Cooper & Haney, 2014; Hernandez-Avila, Rounsaville, & Kranzler, 2004; Hser, Anglin, & Booth, 1987). Accompanying this deleterious course to drug dependence includes both the greater susceptibility to relapse and the increased difficulty in the cessation of drug use in women (Becker & Hu, 2008; Becker & Koob, 2016). In one study using randomly prompted real time data collection procedures, women reported greater levels of cocaine use despite trying to avoid drug use (Kennedy, Epstein, Phillips, & Preston, 2013). This impaired ability to cease drug use is intensified by reports of more robust subjective effects associated with abuse liability, such as drug liking, in women versus men (Cooper & Haney, 2014). The influence of sex upon exposure to drug cues is also observed by elevations in subjective craving reports in women exposed to cocaine cues, when compared to their male counterparts (Kennedy et al.,

2013; Robbins, Ehrman, Childress, & O'Brien, 1999). The cycle of addiction also appears to be more damaging in female compared to male cocaine users, as they are victim to longer intervals of active cocaine use following periods of abstinence (Gallop et al., 2007). This inability to abstain from drug use despite the desire to do so is reflected in the disturbing 20% increase in reported relapse to opiates in females compared to males (Maehira et al., 2013). However, it should be noted that other studies have not reported any differences in opiate relapse across sex, and that these differences might reflect cultural differences between South Asian (Maehira et al., 2013) and Western cultures (Greenfield et al., 2007). Additionally, while the greater incidence of cue provoked relapse to cocaine in women has often been reported (Kennedy et al., 2013; Sinha et al., 2007), the data appear to be separable from findings in opiate users, where elevations in heroin cue cravings may be similar to those reported in male users (Kennedy et al., 2013; Yu et al., 2007). Despite these mixed findings, the overall consensus is that a potentially relevant vulnerability to drug abuse in women exists. Therefore, the current thesis uses female rats to evaluate whether relapse in females is also a function of increased vulnerability to drug cues and environmental challenges.

In laboratory animals, the evidence of sex differences on cocaine taking and seeking are in agreement with the human literature. Specifically, the rate of use, the amount consumed, and the level of cocaine seeking is elevated in female rats compared to male rats (Becker & Koob, 2016; Becker, Perry, & Westenbroek, 2012; Roth, Cosgrove, & Carroll, 2004). Similar to clinical studies, preclinical reports investigating opiates are both sparse and inconsistent. For example, either decreases or no differences are reported in the rates of acquisition, while an increase or no changes in level of consumption are observed (Becker & Koob, 2016; Roth et al., 2004; Venniro, Zhang, Shaham, & Caprioli, 2017). Specifically, Stewart and colleagues (1996), failed to identify meaningful differences among sexes in the rates of heroin self-administration or the reinforcing effects of heroin. However, others have reported that female rats self-administered more heroin and morphine than males (Cicero, Aylward, & Meyer, 2003), and acquired heroin self-administration more rapidly than males (Lynch & Carroll, 1999).

Data gathered on the stark difference in the progression to dependence, and vulnerability to relapse across sexes have remained largely consistent across studies of cocaine users. Similarly, information on sex differences in opiate use and relapse suffers from a great deal of ambiguity, resulting from limited reports on the matter. This inconsistency may result from the lack of control on fluctuating ovarian hormones in surveyed women, and, in studies that have examined sex differences in rodents. One possibility is that the higher vulnerability to drugs of abuse in women, and the inconsistency of data collected in opiate users, may be associated with menstrual cycle-related fluctuations in ovarian hormones (i.e., estradiol [E2] and progesterone).

1.7.2 - *The true masterminds: Do ovarian hormones regulate drug taking and seeking?*

In humans, the positive, subjective effects of smoked cocaine appear to be similar across sexes, with the exception of women in the luteal phase (i.e., high estrogen, high progesterone), where reduced levels of craving, and attenuated positive effects to smoked cocaine and nicotine are reported (Allen et al., 2015; Evans & Foltin, 2010; Goletiani, Siegel, Lukas, & Hudson, 2015). The diminished positive, subjective effects to cocaine in women appear to result from the elevated levels of circulating estrogen, while higher levels of plasma progesterone (i.e., during the mid-luteal phase) have been associated with decreased stress- and drug cue-induced craving (Sinha et al., 2007). Therefore, the levels of circulating ovarian hormones may modulate the subjective and behavioural response to drugs. These data, however, rely on surveyed responses. To our knowledge, no studies have investigated the role of ovarian hormones in women in a controlled manner, as would be the case in women prescribed hormone-based birth control treatments. Studies in which hormones are manipulated in a controlled manner have been conducted using rodents, and mostly focus on stimulant drugs (Becker & Hu, 2008; Cummings et al., 2011; Roberts, Bennett, & Vickers, 1989b).

Following surgical removal of the ovaries (i.e., ovariectomy; OVX), rats show a robust reduction in cocaine self-administration, which can be reversed by treatment with a high dose of E2 (the

most abundant estrogen in the rat; Lynch, Roth, Mickelberg, & Carroll, 2001). The enhanced response to drugs observed during high E2 states is further evidenced by findings that rats work harder for cocaine during the estrus phase (moderate E2, low progesterone), compared to other phases of the estrous cycle (Roberts, Bennett, & Vickers, 1989a). These data suggest that E2 may increase motivation for drug taking, which is consistent with reports that ovariectomized rats show higher responding for cocaine than males, following E2 replacement (Becker & Hu, 2008; Cummings et al., 2011; Roberts, Bennett, & Vickers, 1989b). While female rats will self-administer more cocaine under E2 treatment (Lynch & Taylor, 2005), attenuated cue-induced reinstatement has been reported during estrus, indicating a lower motivational significance to cues during this phase (Fuchs, Evans, Mehta, Case, & See, 2005). This curious contrast in terms of ovarian hormones between drug-taking versus -seeking behaviour requires further study in paradigms that investigate cue manipulations in animal models of relapse.

While E2 appears to have a generally augmenting effect in cocaine studies, the opposite has been observed in response to progesterone treatment. For instance, clinical trials in women have revealed strong negative correlations in the plasma levels of progesterone and the level of stress-induced cocaine craving (Sinha et al., 2007). The attenuating effects of progesterone on estrogen-induced changes in drug subjective properties have also been outlined. Indeed, reductions in the subjective effects, response, and desire to smoke cocaine have been observed during the luteal phase (high estrogen, high progesterone) compared to the follicular phase (high estrogen, low progesterone) of the menstrual cycle (Evans, Haney, & Foltin, 2002; Sofuoglu, Dudish-Poulsen, Nelson, Pentel, & Hatsukami, 1999). In accordance, compared to the luteal phase, women in the follicular phase exhibit enhanced activity within reward-related brain regions following exposure to smoking cues (Franklin et al., 2015).

The disruptive effects of progesterone on drug-related behaviours have been documented in female rodents as well. For instance, progesterone was shown to block cocaine-primed reinstatement (Anker, Holtz, Zlebnik, & Carroll, 2009), and, to disrupt the E2 enhancing effects of cocaine acquisition in ovariectomized rats where concurrent progesterone injections

reduced E2-induced augmentations of cocaine taking (Jackson, Robinson, & Becker, 2006). Moreover, progesterone treatment attenuates cocaine seeking in female rats (Feltenstein, Byrd, Henderson, & See, 2009). Therefore, we contend that E2 may enhance, while progesterone may attenuate, drug seeking in rodents, an effect that has yet to be thoroughly investigated in heroin-seeking female rats.

Reports on the effects of ovarian hormones on opiate self-administration are scarce. The few studies using a heroin self-administration procedure reported similar levels of self-administration in both ovariectomized and free-cycling sham operated rats. Furthermore, high dose E2 replacement was found to facilitate the acquisition and increase the intake of heroin in both groups (Roth, Casimir, & Carroll, 2002). During proestrus (high E2, high progesterone), a ~70% decline in heroin self-administration has also been observed in rats, compared to all other phases of the estrous cycle (Lacy, Strickland, Feinstein, Robinson, & Smith, 2016). This suggests that the enhancing effects of E2 and the attenuating effects of progesterone may extend to heroin self-administration as well. To our knowledge, only one study has investigated the effects of sex on food restriction and heroin self-administration. In this study, female rats demonstrated greater heroin intake than male rats, and food restriction nearly doubled heroin self-administration in both the female and male rats (Carroll, Campbell, & Heideman, 2001).

Sex differences in eating behaviours have also been explored by measuring the impact of ovarian hormones on food intake. For example, E2 reduces food intake, while progesterone has been shown to increase food intake (Culbert, Racine, & Klump, 2016). In ovariectomized rats, E2 injections were found to suppress food intake and decrease body weight (BW), with both effects reversed by injections of progesterone (Wade, 1975). Complementary evidence in humans suffering from bulimia nervosa has also reported that low levels of E2 and high levels of progesterone were associated with increases in binge eating (Edler, Lipson, & Keel, 2007). Thus, it appears that E2 may suppress appetite, that which can then be recovered by the appetite enhancing effects of progesterone.

The investigation of sex hormones in augmented heroin seeking in food-restricted rats may suggest alternate approaches to treatment across sexes. However, there are common underlying neuronal mechanisms that may contribute to drug abuse and the impact of associated cues. Therefore, we have also opted to pursue a neuronal approach to the study of augmented heroin seeking in food-restricted rats.

1.8 - The neuronal approach: Understanding the role of glutamate receptors in food-restriction-induced drug-cue seeking

Drug administration can produce immediate or short-term adaptations to promote the continued use of, and relapse to, the abused drug. However, the path to an addicted brain is thought to constitute a learned habitual response targeted at drug consumption. This habitual response will develop as a result of the perturbations within the neural circuitry responsible for synaptic plasticity, the ability for synapses to strengthen or weaken over time.

By modifying the way in which neurons communicate, drugs of abuse can fundamentally mold drug associated behaviour and increase the risk for relapse. Glutamate transmission has emerged as a critical contributor to the development of drug-induced neuronal plasticity, and the subsequent persistence of drug seeking (Kalivas, 2009; Kalivas, LaLumiere, Knackstedt, & Shen, 2009). The significant role for glutamate in drug addiction is illustrated by the finding that all drugs of abuse can alter glutamate transmission in one way or another. For example, exposure to almost all drugs of abuse can initiate the extracellular release of glutamate within the ventral tegmental area (VTA), a brain structure highly implicated in reward processing (Gass & Olive, 2008; Wise, Bin Wang, & You, 2008; You, Wang, Zitzman, Azari, & Wise, 2007). The long-term adaptions in glutamatergic transmission are also reflected across the different stages of the drug-addicted brain; initiation, maintenance, withdrawal, relapse. In response to acute opiate-exposure, a suppression of glutamate overflow is observed across several brain structures (Sepulveda, Hernandez, Rada, Tucci, & Contreras, 1998). However, following chronic exposure to the same opiates, a neuronal supersensitivity to glutamate can develop (Satoh,

Zieglgänsberger, & Herz, 1976; Sepulveda et al., 1998). The outcome of this supersensitivity may contribute to the increased overflow of glutamate release observed in opiate-withdrawn rats (Aghajanian, Kogan, & Moghaddam, 1994; Sepulveda et al., 1998; Sepúlveda, Oliva, & Contreras, 2004). More relevant to the current thesis is the impact of the glutamatergic adaptations on heroin cue-induced seeking. However, reports on these opiate-glutamate interactions have been rather limited in comparison to studies on psychostimulant-glutamate effects. In one report, inhibiting glutamate transmission was used to demonstrate that glutamate release is *necessary* for cue-induced heroin seeking (LaLumiere & Kalivas, 2008).

In Chapter 3, we explore the enduring system level alterations which underlie the pervasive nature of addiction and sustained vulnerability to relapse long after the cessation of drug use (Shen, Moussawi, Zhou, Toda, & Kalivas, 2011; Thomas, Beurrier, Bonci, & Malenka, 2001). First, a rationale for our decision to focus on the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPAR) will be presented. Next, the role of AMPARs in brain sites associated with the rewarding effects of drugs of abuse will be discussed. Finally, we will examine the impact of caloric restriction and its interaction with abused drugs on AMPARs.

1.8.1 - *Understanding the glutamate receptors.*

It is estimated that 70% of synaptic transmission in the mammalian central nervous system is mediated by glutamate (Gass & Olive, 2008). In the synaptic cleft, glutamate exerts its effects by acting on one of two classes of receptors: ionotropic (iGluR) or metabotropic (mGlur) glutamate receptors. The iGluRs are ligand-gated ion channels that are thought to mediate fast, excitatory neurotransmission, and are mostly located post-synaptically. The mGluRs, located both pre-and post-synaptically, are excitatory G-protein coupled receptors that resolve slower synaptic processes.

Although the role of iGluRs and mGluRs are pertinent to the effects of drug taking and seeking in animal models, we have chosen to narrow our focus to the alterations of the iGluRs. Specifically, we are interested in the immediate effects of cue exposure in rats, which requires short, fast acting alterations, and are most likely to target iGluRs. However, we also note the importance of mGluRs, and refer the reader to a comprehensive review of mGluRs and their role in addictive behaviours (Pomierny-Chamioło et al., 2014).

The actions of iGluRs are achieved by one of three receptor families: N-methyl-D-aspartate (NMDA), kainate, and AMPARs which constitute distinct subunit compositions. The experiments presented in Chapter three investigated solely the role of AMPARs, given the overwhelming evidence of their involvement in drug addiction (Bowers, Chen, & Bonci, 2010; D'Souza, 2015) and in food-restriction-induced neuroplasticity (Ouyang et al., 2017). Our rationale for this focus is further supported by the substantial role of AMPARs in neuronal plasticity (S. Jones & Bonci, 2005; Santos, Carvalho, Caldeira, & Duarte, 2009).

Composed of a combination of GluR1-4 subunits, AMPARs possess a heterotetrameric protein complex architecture. This forms ligand-gated channels which are activated following glutamate binding (Dingledine, Borges, Bowie, & Traynelis, 1999), resulting in receptor permeability to a variety of cations including calcium, sodium and potassium. The actions of AMPARs is thought to be intertwined with their specific subunit compositions (Collingridge, Isaac, & Wang, 2004; Derkach, Oh, Guire, & Soderling, 2007). The presence or absence of the GluA2 receptor subunit is of particular interest, as it determines the calcium permeability of AMPARs. Only GluA2-lacking AMPARs, which constitute a small proportion of AMPARs in the brain, are permeable to calcium due to the absence of arginine within the ion pore channel (Derkach et al., 2007; Gass & Olive, 2008; Hollmann, Hartley, & Heinemann, 1991; Vandenberghe, Robberecht, & Brorson, 2000).

The phosphorylation and trafficking of the AMPAR subunits to and from the cell surface are also believed to contribute heavily to the receptor functions. It has been suggested that the induction of synaptic plasticity increases the phosphorylation of GluA1 (Mammen, Kameyama, & Roche, 1997), highlighting an important role for the phosphorylation of GluA1 in neuronal plasticity. Accordingly, in mice lacking the GluA1 serine (Ser) phosphorylation sites (Ser 831, Ser 845, and Ser 818) perturbations in the induction of synaptic plasticity are observed (Lee et al., 2003; Santos et al., 2009).

Targeted pharmacological interventions have also demonstrated an interesting duality to the role of AMPARs in drug abuse. Indeed, activation of AMPARs may enhance, while inactivation may disrupt, drug taking. For example, systemic administration of piracetam[1], a positive allosteric modulator (PAM) of the AMPAR, facilitated a heroin-induced CPP in rats (Xu et al., 2015). In contrast, rats administered AMPA antagonists demonstrated reduced cocaine (Pierce, Meil, & Kalivas, 1997) and alcohol taking (Stephens & Brown, 1999). However, the effects of AMPARs appear to be region specific. For example, agonism of AMPARs may increase or decrease drug seeking, depending on the specific brain targets. As such, we explore the effects of AMPARs across several reward-related brain structures below.

1.8.2 - The interplay of AMPARs and drug actions within the nucleus accumbens.

In part, glutamate and AMPARs within the nucleus accumbens (NAc) are hypothesized to regulate the attribution of salience to drug cues and the motivation for reward-seeking and taking (Hyman, Malenka, & Nestler, 2006; Kalivas & Volkow, 2005; Robinson & Berridge, 1993). Due to the lack of data examining heroin-related behaviours, we explore this AMPAR regulation with respect to rats exposed to psychostimulants. For instance, AMPA infusions into the NAc were shown to potentiate cocaine-induced reinstatement, while antagonism of AMPARs prevented this reinstatement (Cornish & Kalivas, 2000; Ping, Xi, Prasad, Wang, & Kruzich, 2008). These data demonstrate that a direct manipulation of AMPARs within the NAc can have

[1]Although lacking any concrete evidence, it is interesting to note that the rise in the inclusion of piracetam to street drugs may enhance the subjective effects experienced and allow distributers to reduce their purity.

powerful, immediate effects on the expression of cocaine seeking. When investigating the role of cocaine on changes within the glutamatergic system, repeated, but not acute, injections of cocaine were shown to induce synaptic plasticity (Kourrich, Rothwell, Klug, & Thomas, 2007). Pertinent to this discussion, however, is that the potentiation of AMPAR-mediated synaptic transmission only occurs following prolonged withdrawal. This is further supported by evidence that cell surface expression in NAc AMPARs is only observed following prolonged withdrawal from cocaine (Boudreau & Wolf, 2005; Kourrich et al., 2007; Loweth, Tseng, & Wolf, 2014; Reimers, Milovanovic, & Wolf, 2011).

The role of NMDARs in neuronal plasticity cannot be disputed. However, we focused our attention on the role of AMPARs given some indications that NMDAR transmission remains unaltered following both acute and prolonged withdrawal from cocaine. Furthermore, any decrease in AMPAR/NMDAR ratios observed was posited to reflect disruptions in AMPAR transmission alone (Thomas et al., 2001). Considerable data have been collected on the role of AMPARs in response to cocaine administration. Interestingly, a depression of excitatory neurotransmission has been reported during early withdrawal from cocaine self-administration (Schramm-Sapyta, Olsen, & Winder, 2006), while enhanced AMPAR transmission was detected after prolonged withdrawal (Conrad et al., 2008). Specifically, this elevation in AMPAR transmission is thought to result from synaptic insertion of GluA2-lacking AMPARs in the NAc (Conrad et al., 2008). Furthermore, synaptic AMPARs also appear elevated in the NAc after prolonged withdrawal in rats with a history of cocaine self-administration (McCutcheon, Wang, Tseng, Wolf, & Marinelli, 2011; Purgianto et al., 2013). In cocaine-treated rats, medium spiny neuron (MSN) NAc surface and intracellular GluA1 levels were increased, and higher conductance in these AMPARs was observed 45 days after the cessation of the drug (Conrad et al., 2008). Our focus on GluA1 AMPARs and the rationale for the experiments described below (Chapter 3) relates to evidence showing GluA2 surface and intracellular levels may not be changed following prolonged withdrawal in cocaine-treated rats (Conrad et al., 2008). It is therefore evident that cocaine use can critically impact the functioning of the GluA1 subunit

AMPAR. While this direct influence is significant, our interest pertains to the indirect impact of abused drugs through their associated cues.

One consideration is that enhanced cue-induced drug seeking results from the increased responsiveness of NAc MSNs to drug cues. Conrad and colleagues (2008) have argued that this enhancement likely results from the potentiation of AMPARs within the NAc. Following treatment with a selective GluA2-lacking AMPAR blocker in the NAc shell, they reported an immediate decreased cocaine-seeking in rats subjected to a prolonged withdrawal period (Conrad et al., 2008). In the NAc core region, re-exposure to cocaine cues was found to promote elevations in markers associated with increased synaptic transmission(e.g., AMPAR/NMDAR ratio), and the dendritic spine density of MSNs (Gipson et al., 2013). Such effects were reported within 15 minutes following cue exposure, suggesting a rapid cue-induced synaptic potentiation in the NAc core MSNs. Therefore, it is likely that they may contribute to the increase in cocaine seeking observed following cue exposure. These data suggest the strong possibility that changes in AMPAR levels may be visible following a one hour drug-seeking test using our procedure.

When studying heroin seeking, there are several caveats that must often be considered. A specific example is that evidence on the underlying mechanisms of drug abuse and the role of glutamate in the NAc has largely focused on rats exposed to psychostimulants. However, there exist some indications that extracellular glutamate levels and subunit expression changes occur following exposure to morphine (Glass et al., 2008; Hearing et al., 2016). The fact that these differences may contrast evidence from cocaine studies is quite compelling, and further supports the importance of extending studies to opiate use. For instance, following both chronic cocaine self-administration and withdrawal, an increase in cell surface expression of GluR1 subunit AMPARs within the NAc and disrupted long-term potentiation (LTP) induction were found (Chen, Hopf, & Bonci, 2010). As outlined above, this is suggestive of enhancements in synaptic plasticity. In contrast, chronic passive (non-contingent) morphine exposure resulted in reduced cell surface level expression of AMPARs in the NAc (Glass et al., 2008). These

findings make it difficult to draw comparisons in glutamate receptor (GluA-R) functioning across drug classes, therefore we must practice caution when generalizing evidence from studies on psychostimulant use.

1.8.3 - *The interplay of AMPARs and drug actions within the medial prefrontal cortex.*

It is now accepted that the addicted brain is associated with reductions in basal medial prefrontal cortex (mPFC) activity (van Huijstee & Mansvelder, 2014; Volkow, Fowler, & Wang, 2003), which is also the case following self-administration of abused drugs in animals (Sun & Rebec, 2006). The mPFC, which heavily projects glutamatergic signals to the VTA, amygdala, and NAc, is composed of dorsal (i.e., prelimbic) and ventral (i.e., infralimbic) regions, including heterogenous anatomical and functional characteristics (Gipson, Kupchik, & Kalivas, 2014; Vertes, 2004). An important distinction is that the dorsal sub-region projects to the NAc shell and is thought to be involved in the initiation of cue-induced relapse. Alternatively, the ventral sub-region projects to the NAc shell, and is thought to suppress drug seeking (Gipson et al., 2014; Ma et al., 2014; Shin et al., 2018).

Studies on adaptations in AMPAR subunits following heroin exposure have been somewhat inconsistent. For example, one study has reported no changes in the AMPAR GluA1, but rather a significant down regulation of the GluA2 subunit membrane expression after heroin self-administration (Van den Oever et al., 2008). In contrast, Hearing et. al. (2016) found increases in AMPAR expression in the NAc, 10-14 days following the cessation of repeated experimenter-administered morphine. During a reinstatement test, this increase was reversed following infralimbic cortex (IL) optogenetic stimulation, designed to induce long-term depression (LTD; Hearing et al., 2016). Collectively, these data elegantly demonstrate that synaptic plasticity within the IL-NAc pathway may play a critical role in opiate reward. Furthermore, the finding that AMPARs are significantly involved in heroin seeking is also demonstrated following treatment with the allosteric AMPAR potentiator, PEPA, into the IL. Specifically, the administration of PEPA 14 days following the extinction of heroin cues resulted in a degradation

of the heroin-seeking response (Chen et al., 2016). More so, this behavioural decrease was accompanied by the enhanced expression of GluA1 within the IL and NAc (Chen et al., 2016).

Other studies have suggested the exclusive involvement of the prelimbic cortex (PL) in drug-related behaviours. For example, Jaegar and colleagues (2013) demonstrated that intra-PL, but not intra-IL, infusions of a non-specific AMPAR antagonist can facilitate CPP in rats receiving a normally sub-reward threshold conditioning dose of morphine. To further complicate the matter, a recent report has indicated that under certain tasks, mPFC AMPARs do not affect drug taking (Gutman, Ewald, Cosem, Worth, & LaLumiere, 2016). Indeed, during a discriminative stimulus task, responses in the presence of a cocaine- or non-drug-paired-cue were not affected by intra-PL or intra-IL infusions of an AMPAR antagonist (Gutman et al., 2016). We do note that the discrepancy between studies may involve the effects of experimenter- versus self-administred drug protocols. However, the role of AMPARs in the mPFC remain unclear, and may depend on the specific reward-related task.

Glutamatergic transmission, particularly through AMPARs, may play a substantial role in mediating drug-seeking and -taking behaviours. In addition, it is evident that these effects are not only a consequence of drug use but are applicable in the exposure to drug cues following prolonged, but not necessarily acute, withdrawal. Given the aforementioned studies, we confidently focus on alterations in the AMPARs GluA1 subunit in the NAc and mPFC in the experiments described in Chapter 3. We have yet, however, to discuss the role of these receptors in response to dietary manipulations, which remains a core facet of our procedure.

1.8.4 - What about hunger? The impact of caloric restriction on AMPARs, and the interplay with drugs of abuse.

The potential role for glutamate and AMPARs in regulating the enduring impact of abused drugs also extends to caloric restriction, and its interplay with drugs of abuse. Under food restriction, exposure to a cocaine- or morphine-paired environment has been shown to elevate

GluA1, p-GluA1, and GluA2 levels in the NAc core in cocaine-treated (Zheng, Cabeza de Vaca, Jurkowski, & Carr, 2015), and the NAc shell in morphine-treated rats, respectively (Jung et al., 2016). Using a lateral hypothalamic self-stimulation procedure, where rats are trained to self-administer electrical stimulation within the lateral hypothalamus, *d*-amphetamine reduced the rewarding threshold of the stimulation to a greater degree in food-restricted versus sated rats (Peng, Cabeza de Vaca, Ziff, & Carr, 2014). This effect was also associated with greater GluA1, p-GluA1, and GluA2 levels in the NAc (Peng et al., 2014). The effects of brain stimulation reward (BSR) were believed to be managed through changes in the GluA1 AMPARs specifically, as the administration of the selective GluA1 receptor antagonist 1-naphthylacetylsperimine (Naspm) was found to reverse this reward reducing threshold in food-restricted rats exclusively (Peng et al., 2014).

In several experiments from Carr's group (Carr, 2016; Ouyang et al., 2017; Peng et al., 2015), food-restricted rats assigned to control conditions (i.e., drinking water instead of sucrose, treated with saline and not *d*-amphetamine) routinely exhibited elevated levels in GluA1, but not GluA2, in the NAc when compared to control rats with unrestricted access to chow. This complements findings in which experimentally naïve food-restricted rats (housed in home cages), showed greater protein concentrations of p-ser845-GluA1 and GluA1, but not GluA2 or GluA3 relative to sated rats (Carr, 2016). As GluA1 and p-Ser845-GluA1 levels appear to be consistently elevated following chronic food restriction, it is therefore conceivable that food restriction may potentiate the rewarding effects of appetitive stimuli by enhancing the synaptic incorporation of calcium permeable GluA1 AMPARs into the cell surface.

An understanding of the underlying mechanisms of drug addiction is integral for the development of effective treatment. Therefore, the interpretation of behaviour in the study of these neuronal mechanisms is also critical to our translation from animal models to human condition. Next, we introduce our final approach, which focused on identifying the motivational behavioural properties of heroin-associated cues in food-restricted rats.

1.9 - The behavioural approach: Understanding the modulation of cue value in the food-restriction-induced augmentation of drug-cue seeking

1.9.1 - Learning the importance of drug cues.

In humans, environmental stimuli can acquire motivational significance through repeated pairings with abused drugs, which can subsequently drive drug-taking and -seeking behaviours. More so, exposure to these drug-associated cues can precipitate a return to drug seeking, even after long periods of withdrawal[2] from drug taking (Gawin & Kleber, 1986). To understand the processes that govern the output of drug-seeking behaviour, we first examine the potential mechanisms that determine its maturation.

Learning theories of addiction link the development of drug seeking following self-administration to two processes. The first is an associative process in which an operant behaviour or action (A) is paired with the delivery of the drug or outcome (O). The second process involves the learning of a stimulus-response association. Here, the pharmacological effects of the drug reinforces the association between the response and stimuli present at the time of reinforcement (Everitt & Robbins, 2016). Everritt and Robbins (2016) argue that these stimulus-response associative processes contribute to the development of habitual responding for abused drugs, which they consider separate processes. This is further supported by findings that the devaluation of a primary reinforcer (e.g., drug) following the formation of a drug-cue association did not affect responding in cue-induced drug seeking (Zapata, Minney, & Shippenberg, 2010). Importantly, this suggests that responding for a drug-associated cue entails a habitual behaviour separable from the reinforcing properties of the drug itself. This dissociation highlights the importance attributed to drug-associated cues, and the difficulty in suppressing the motivation to seek such cues in the presence of negative consequences associated with drug use.

[2]The term withdrawal will be used to refer to any periods of drug cessation. We avoid here the term abstinence as abstinence by definition implies a self-enforced behaviour, which is often not the case within animal models. Abstinence will only be used when the cessation of drug taking is self-enforced. Investigations concerning the effects of physical drug withdrawal in animals will be specifically referred to using those terms.

The incentive sensitization theory of addiction also needs to be emphasized. This theory argues that stimulus-response learning does not address the pathological motivation for drugs that extends beyond habit formation (Robinson & Berridge, 2008). In fact, this theory posits that the stimulus-outcome association is critically involved in driving drug seeking in the absence of the drug. As previously neutral stimuli become imbued with incentive motivational properties following repeated pairings with a drug reward, the stimulus-outcome association becomes the main trigger for drug seeking (Berridge, Robinson, & Aldridge, 2009; Robinson & Berridge, 2008; see Chapter 5 for further details). In agreement with this, recent work has also indicated that the formation of a habitual response may not be necessary for drug seeking (Singer, Fadanellie, Kawa, & Robinson, 2018).

1.9.2 - *The enhancing effects of cues on drug taking and seeking.*

In animal models of relapse, the capacity for drug-paired cues to augment drug taking and seeking has been demonstrated using a wide variety of drug classes, including heroin, cocaine, nicotine, and alcohol (Venniro, Caprioli, & Shaham, 2016). These cue-induced drug enhancing effects are particularly evident in nicotine self-administration procedures. Here, the rate of acquisition of an operant response reinforced with nicotine delivery was boosted when nicotine delivery was paired with a visual stimulus (Caggiula et al., 2002). This interplay between nicotine and its associated cues also extends to stimulants (i.e., cocaine, *d*-amphetamine), where drug-paired cues also increase a rat's willingness to respond for the drug itself (Deroche-Gamonet, Piat, Le Moal, & Piazza, 2002; Keller, Vollrath-Smith, Jafari, & Ikemoto, 2014). These strong motivational properties of drug-associated cues are also demonstrated using the cue-induced reinstatement procedure. The reintroduction to an environment previously paired with heroin or cocaine, and exposure to discrete or discriminative drug-associated cues have all been shown to successfully reinstate extinguished drug seeking (Crombag, Bossert, Koya, & Shaham, 2008a; Lee, Milton, & Everitt, 2006; Shalev et al., 2002; Stewart, 2000). Importantly, the reinstatement of heroin or cocaine seeking has been demonstrated long after physical

withdrawal from the drug has diminished, suggesting a strong persistence of drug craving perpetuated by such cues (Grimm, Hope, Wise, & Shaham, 2001; Pickens et al., 2011; Venniro et al., 2016).

1.9.3 - *The inherently enhancing effects of cues ~~on drug taking and seeking~~.*

As previously mentioned, the effects of drug cues in animal models of relapse have been extensively examined. However, these discussions often omit the influence that cues have irrespective of their association with the abused drug. Self-administration procedures commonly pair multi-modal cues, consisting of both visual and auditory components with the pharmacological effects of the drug. Following extinction or withdrawal from the drug, drug seeking initiated by re-exposure to these drug-paired cues is considered a measure of drug craving, or a measure of the motivation to seek a drug in animals. Interestingly, however, the inherent reinforcing properties of the visual stimulus are often ignored. For instance, in a choice procedure in which one lever was paired with nicotine delivery and another lever was paired with a visual stimulus, greater responding for the visual stimulus was recorded. Furthermore, this responding was similar to responses resulting from a combination of nicotine and the stimulus in a one lever procedure (Palmatier et al., 2006). Data have also demonstrated that responding for nicotine or methylphenidate paired with a visual stimulus is augmented when compared to non-paired presentations (Marusich, Beckmann, Gipson, & Bardo, 2011). This suggests that visual stimuli can act as weak reinforcers. In a set of reports by Sved's group investigating the role of a visual reinforcer using a nicotine-self-administration procedure, it was suggested that nicotine acts both as a weak primary reinforcer and as an enhancer of the reinforcing effects of a non-pharmacologically-paired visual stimulus (Chaudhri, Caggiula, Donny, Booth, et al., 2006a; Chaudhri, Caggiula, Donny, Palmatier, et al., 2006b; Palmatier et al., 2006). These reports corroborate earlier work which indicated that rodents will respond for light-reinforcement alone (Morris, Crowder, & Crowder, 1961; Stewart, 1960). Collectively, these studies highlight the intricate interplay between drugs and cues, and the necessity for a

clearer understanding of their roles in animal models of relapse such as those presented within the current thesis.

The persistence of habitual responding is thought to be greatly influenced by underlying motivational processes. One such motivational process includes the attribution of incentive motivational properties to drug cues that may contribute to persistent responses in the absence of the drug. In this thesis, one of our primary goals was to investigate the modulation of heroin-associated cue value by dietary restrictions that may influence the motivational process that contribute to drug seeking. Therefore, we next examine the influence of dietary manipulations on visual cues.

1.9.4 - *The influence of dietary manipulations on cues in animals studies.*

In animals, the interaction between caloric restriction and drugs of abuse has been demonstrated using a variety of paradigms including self-administration (Carroll & Meisch, 1984), CPP (Bell, Stewart, Thompson, & Meisch, 1997; Jung et al., 2016; Zheng et al., 2015), and electrical BSR (Cabeza de Vaca & Carr, 1998) procedures. In chronically food-restricted rats, the effects of caloric restriction on the motivational properties of non-food related conditioned reinforcers have also been examined, where reduced thresholds for electrical BSR have been observed (Fulton, Woodside, & Shizgal, 2000). More recently, Zheng and colleagues (2012) reported that food restriction enhanced cocaine CPP, established under non-restricted conditions. These data are consistent with the idea that food restriction acts to modulate drug-related behaviours in such a way as to disproportionally influence drug self-administration. Such findings also lend further support for the conditioned reinforcing properties of abused drugs (Carr, 2007; Stuber, Evans, Higgins, Pu, & Figlewicz, 2002). Research from our laboratory justifies this interpretation, given that chronically food-restricted rats under withdrawal exhibit a robust augmentation in heroin seeking (D'Cunha, Sedki, Macri, Casola, & Shalev, 2013). It remains unclear, however, whether this enhanced drug seeking results from non-specific motivational/attentional processes triggered by the hunger state (Sedki, Abbas, Angelis,

Martin, et al., 2013a), or due to a more specific augmentation of incentive motivational properties of the drug cues. Therefore, one approach is to investigate whether the incentive motivational properties of heroin cues increase following chronic exposure to food restriction. This question needs to be addressed carefully, however, in order to measure whether caloric restriction enhances the inherent motivational value of all cues, irrespective of their drug-conditioned reinforcing properties.

In the late 1950s and early 1960s, several studies set out to dissect the role of hunger on the reinforcing value of drug-paired cues. Most (Davis, 1958; Hurwitz & De, 1958; Segal, 1959; Stewart, 1960), but not all (Hurwitz & De, 1958; Smith & Donahoe, 1966) reported higher lever pressing for a visual stimulus in hungry rats. In a more recent attempt to investigate this phenomenon, Keller and colleagues (2014) reported that food-restricted rats treated with *d*-amphetamine exhibited a robust potentiation in lever responding in the presence, compared to the absence, of a visual stimulus. However, investigations into the effects of visual stimuli using opiate-seeking procedures and in calorically restricted rats, remain even more scant. We contend that the lack of research within this area is harmful for the validity of animal models using visual stimuli to drive drug-seeking behaviours. Furthermore, the generalizability of reports that have not adopted the necessary behavioural controls should be interpreted with caution. Without the inclusion of fundamental control conditions, it is difficult to differentiate behavioural responses to visual stimuli as *cue seeking* or *drug seeking*. Therefore, we believe that it remains unclear whether the effects of hunger on *drug seeking* result from an increased attribution of incentive motivational properties to drug-paired cues, or to the inherent motivational salience of visual cues.

1.9.5 - How to study motivation in animals.

In rodents, the most ubiquitous measure of reward motivation may be by evaluating responses under a progressive ratio (PR) schedule of reinforcement (Arnold & Roberts, 1997). Initially developed to evaluate the reinforcing efficacy of natural rewards, the PR schedule of

reinforcement was later adapted for intracranial self-stimulation (ICSS) and drug self-administration paradigms (Arnold & Roberts, 1997; Hodos, 1961). Under this schedule, the number of operant responses required to earn the reinforcer increases following each delivery of the reinforcer, according to an exponential progression derived from the formula PR cost = $[5e^{(\text{reward number X cost})}]$-5 (Richardson & Roberts, 1996), rounded to the nearest integer. The number of rewards earned, known as the *breakpoint*, is believed to be rate independent, and is therefore a more accurate measure of reinforcing efficacy. While there are clear advantages for the use of PR schedules, responding under these schedules is almost exclusively driven by primary reinforcers. To our knowledge, research on the efficacy of cue-induced operant responding reinforced under a PR schedule continues to be limited. Therefore, we have initiated the use of different approaches to better understand the motivational properties of drug cues.

The ability for drug cues to support new learning in the absence of primary reinforcement, is thought to reflect the motivational properties that may support drug seeking. Furthermore, the conditioned reinforcing properties of cues are difficult to dissociate from the primary reinforcing effects of drugs as the same operant response (i.e., a lever response) is used to acquire both (Di Ciano, Robbins, & Everitt, 2008). An effective approach to assess changes in the conditioned reinforcing properties of drug-associated cues may be to measure the acquisition of a novel behaviour that is driven solely by drug cues. This can be particularly informative as it addresses response data that may isolate the value of the drug cues from the influence of the primary reinforcer. Evidence using this procedure has shown that cues associated with cocaine can indeed support the acquisition of a novel instrumental response, and that this behaviour persists across multiple tests (Di Ciano et al., 2008; Samaha, Minogianis, & Nachar, 2011). However, these data fail to acknowledge whether responses are due to the reinforcing value of the drug cues or the inherent value of the cues alone. In the current thesis, we utilized both procedures to investigate the motivational properties of drug cues in food-restricted rats, and to answer whether any difference in the incentive value of the cues simply resulted from their inherent rather than conditioned value.

1.10 - Rationale for the current studies: A three-pronged approach

The studies presented below employed a novel procedure to investigate the behavioural, neuronal, and hormonal processes that underlie the augmentation of heroin seeking in chronically food-restricted rats. Our first approach (Chapter 2) was motivated by the ambiguous nature of, and limited data on, the effects of sex differences in heroin seeking, and food restriction in rodents. Therefore, we aimed to elucidate the role of ovarian hormones in the augmentation of heroin seeking in chronically food-restricted female rats. Generally, it is thought that E2 may augment drug seeking and suppress feeding in rats. Given that food restriction augments heroin seeking, then E2-treated, ovariectomized rats may demonstrate greater levels of drug seeking. In contrast, if the appetite suppressing effects of E2 act to reduce hunger and underlie the food-restriction-induced augmentation of heroin seeking, then the motivation to seek heroin-associated cues may be reduced. Additionally, we consider the role of progesterone, which has been shown to attenuate drug seeking and to promote food intake. In Chapter 3, we used a neuronal perspective to investigate how food restriction, compared to unrestricted access to chow, may differentially modulate the expression of GluA1 and p-Ser845-GluA1 AMPAR subunit expression in the NAc and mPFC. Specifically, we investigated their role following prolonged withdrawal and in response to heroin-associated cues. Both receptor and brain targets were selected for several reasons. First, the phosphorylation of the GluA1 receptor appears to be necessary for the expression of food-restriction-enhanced drug seeking, and second, for the trafficking of the receptors to their active site. Third, evidence in our laboratory has indicated a relevant role for NAc dopamine (DA)-D1 receptors (D1-Rs) in the augmentation of heroin seeking in food-restricted rats, a receptor whose downstream effects include the activation and trafficking of GluA1 subunit receptors. Lastly, many studies have remarked on the relevance of the NAc and mPFC in drug addiction and energy balance, and their necessity for behavioural expression of drug-seeking behaviours. For such reasons, we have these as prime targets in our current investigation. In Chapter 4, a behavioural approach was applied to dissect the conditioned rewarding properties of discrete heroin-associated cues

in chronically food-restricted rats using both the PR and novel operant response learning strategies. To account for the inherent motivational properties of visual stimuli, we also investigated food-restriction-induced differences in choice between heroin and light seeking to remove any ambiguity concerning the motivational targets of our food-restricted rats. In this Chapter, our aims were to assess whether the value of the heroin cues was augmented by food restriction, and to ensure that this augmentation was targeted specifically at heroin-associated cues.

Collectively, these studies will further explore the behavioural, neuronal, and hormonal facets underlying the novel procedure developed in our laboratory. Evidence of such nature will authenticate its validity and that of others that use self-administration. Furthermore, these data will clarify different neuronal underpinnings to direct future research and establish the potential role for hormonal treatments in female subjects.

Chapter 2: Food-restriction-induced augmentation of heroin seeking in female rats: manipulations of ovarian hormones

2.1 ABSTRACT

Rationale. Food restriction augments heroin seeking in chronically food-restricted male rats under withdrawal, an effect not yet examined in female rats. Importantly, women and female rats possess an increased vulnerability to drugs of abuse, which may be mediated by fluctuations in ovarian hormones.

Objectives. We investigated the role of estradiol and progesterone in augmented heroin seeking in chronically food- restricted female rats, under withdrawal.

Methods. Female rats self-administered heroin for 10–12 days and were then allowed unrestricted (sated) or restricted access to food (FDR; ~10 % reduction in BW) for 14 days. On day 14, rats underwent a heroin-seeking test. Exp. 1: Rats underwent ovariectomy or sham surgery and were treated with a low dose of estradiol (5.0 % in cholesterol; subcutaneous capsule). Exp. 2: Rats underwent ovariectomy and were administered with a high dose of estradiol (0.5 mg/kg; subcutaneous) for 8 days before testing. Exp. 3: Progesterone injections (2.0 mg/kg; subcutaneous) were administered 24 h and 2 h before testing.

Results. Food restriction resulted in augmented heroin seeking, compared to sated controls. While ovariectomy had no effect, estradiol replacement attenuated the food restriction effect. Injections of progesterone had no effect on heroin seeking in either the sated or FDR groups.

Conclusions. The effect of food restriction on heroin seeking in female rats under withdrawal is as robust as previously found in males. Interestingly, estradiol replacement, but not progesterone, attenuates the food restriction effect in the ovariectomized rats, possibly due to its anorexic properties.

Keywords Food restriction · Heroin seeking · Estradiol · Progesterone · Withdrawal

2.2 INTRODUCTION

Disruptions in energy balance (e.g., restricted food intake) can strongly affect drug-motivated behaviours in humans and animals (Carr, 2007; Cheskin et al., 2005; Franklin et al., 1948; Hanna & Hornick, 1977; Macenski & Meisch, 1999). For example, an increased risk for relapse among calorically restricted abstinent smokers (Hall et al., 1992), as well as an association between moderate caloric restriction and increases in cigarette consumption, (Cheskin et al., 2005) have been reported. Furthermore, a positive correlation was found between the severity of the calorie-intake restriction and the risk of drug taking in young women (Krahn et al., 1992)y, and Pisetsky et al. (2008) reported a strong association between inappropriate weight control behaviours and substance use in high school students. In laboratory animals, the effects of dietary manipulations on drug-related behaviours have been unequivocally demonstrated. For example, the initiation and maintenance of drug self-administration is reliably enhanced following periods of food deficiency in the rat (Carroll et al., 2001; Lu, Shepard, Scott Hall, & Shaham, 2003; Piazza & Le Moal, 1998). Carr (2007) has demonstrated an augmentation of stimulant-drug-induced decreases in electrical BSR threshold in chronically food-restricted rats. Finally, we recently reported a robust augmentation of heroin seeking in chronically food-restricted male rats under withdrawal (D'Cunha et al., 2013; Sedki, D'Cunha, & Shalev, 2013b).

Animal studies examining the effects of caloric restrictions on drug seeking have primarily focused on male subjects. Yet clinical studies indicate significant sex differences in drug addiction. While the rate of drug dependence is greater in men (Brady & Randall, 1999), women tend to escalate their use of heroin and cocaine more rapidly, and become addicted to alcohol, heroin, and cocaine faster compared to men (see reviews by Becker & Hu, 2008; Lynch et al., 2002). With respect to relapse, women tend to exhibit shorter and fewer cocaine-free periods (Griffin, Weiss, Mirin, & Lange, 1989), and are more likely to attribute relapse to stressful life events or depression (Lynch et al., 2002). It is thought that this higher vulnerability to drugs of abuse in women may be associated with menstrual cycle-related fluctuations in ovarian hormones (i.e., estradiol and progesterone). For example, the subjective response to cocaine is lower in the luteal phase, when progesterone levels are elevated, compared to the follicular

phase, when estradiol levels rise gradually (Evans & Foltin, 2010). In addition, it has been reported that higher plasma progesterone (during the midluteal phase) is associated with decreased stress- and drug cue-induced craving (Sinha et al., 2007).

Sex-related changes in drug taking and seeking have been well documented in rodent studies. Compared to male rats, females self-administer more of a variety of drugs, including cocaine (Lynch & Carroll, 1999), ethanol (Lancaster & Spiegel, 1992), and nicotine (Donny et al., 2000). Female rats also demonstrate enhanced cocaine-primed reinstatement compared to male rats (Kippin et al., 2005), as well as greater cocaine seeking following protracted withdrawal (Kerstetter et al. 2008).

An association between cyclic ovarian hormone fluctuations and responses to drugs (Becker & Hu, 2008; Evans & Foltin, 2010) is supported by investigations of the effects of direct manipulation of these hormones. For example, treatment with estradiol increased cocaine acquisition in ovariectomized rats, and concurrent progesterone injections reduced the estradiol-induced augmentations of cocaine taking (Jackson et al., 2006). Moreover, progesterone treatment has been shown to attenuate cocaine seeking in female rats (Feltenstein et al., 2009). Thus, estradiol may enhance, while progesterone may attenuate drug seeking in rodents.

In the only study that investigated the interaction between sex and food-restriction-induced augmentation of heroin self-administration, Carroll et al. (2001) reported an interaction between sex, feeding conditions, and stress response. To our knowledge, there are no reports on the effects of ovarian hormones on food-restriction-induced augmentation of drug taking and seeking. Thus, here, we manipulated the circulating levels of estradiol and progesterone in chronically food- restricted female rats, and assessed heroin seeking following a withdrawal period.

2.3 METHODS

2.3.1 Subjects

A total of 110 female, Long-Evans rats (Charles River, St. Constant, Quebec, Canada; 250–300 g) were used in three experiments. Before surgery, animals were pair-housed for 1 week in the animal care facility (ACF) under reverse light/ dark conditions (lights OFF at 0900 h). Following intravenous (IV) catheterization and 2 days of recovery, rats were single- housed in plastic shoebox cages before being transferred to operant-conditioning chambers for drug self-administration. Subsequently, rats were returned to the ACF and single- housed in shoebox cages for 1 drug-washout day. Next, rats in exp. 1 and 2 (with exception of the SHAM group in exp. 1) underwent bilateral ovariectomy surgery and were allowed 2 days of recovery. Except when noted otherwise, 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.

2.3.2 Surgical procedures

Intravenous catheterization

Rats were implanted with i.v. silastic catheters (Dow Corning, Midland, USA) under xylazine + ketamine (13.0 + 90.0 mg/kg; i.p.) anesthesia, as described previously (Sedki, D'Cunha, & Shalev, 2013b). Following surgery, rats were administered 0.9 % saline (2 ml/ rat; s.c.), ketoprofen (5 mg/kg/rat; s.c.), and penicillin (450, 000 IU/rat; s.c.) for hydration, to reduce pain, and to prevent infection. Rats received two additional ketoprofen injections for 2 days following surgery. Following 24 h of recovery, catheters were flushed daily with heparin + gentamicin in sterile saline (7.5 IU + 12.0 mg/rat) to prevent blockage and infection.

Ovariectomy

Rats in exp. 1 and 2 were anesthetized with an isoflurane-oxygen mixture (induction 4 %; maintenance 2 %) and underwent bilateral ovariectomy (OVX) via a lumbar incision. Rats in the SHAM surgery group underwent an identical surgical procedure, however, their ovaries remained intact. During the same surgery, rats in exp. 1 were also subcutaneously implanted with a 17β-estradiol (E2; Sigma Chemical Co., St. Louis, M) or cholesterol hormone capsule at

the nape of the neck through a 0.5-cm incision. Post-surgical procedures were identical to those that took place following IV surgery.

2.3.3 Drug

Heroin HCl (a contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in sterile saline (5.0 mg/ml) and then further diluted with saline, for each rat according to BW to yield 0.1 mg/kg/infusion. Estradiol (E2), progesterone (P), sesame oil, and cholesterol were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). 17β-estradiol 3-benzoate (EB) was purchased from Steraloids (Newport, RI).

2.3.4 Hormone treatment

Experiment 1. Hormone capsules were assembled using silastic tubing (length 1 cm; ID 1.47 mm; OD 1.96 mm), and sealed with Marine Silicone. The E2 capsules were comprised of 5 % E2 in cholesterol (CH), previously shown to produce E2 serum levels of approximately 37 pg/ml (Almey, Hafez, Hantson, & Brake, 2013). These levels are similar to the naturally circulating low levels of E2 as observed during the estrus phase of the rat estrous cycle (Butcher, Collins, & Fugo, 1974). Capsules that contained 100 % cholesterol were used as the control treatment.

Experiment 2. In order to reliably obtain high plasma levels of estradiol, EB was dissolved in reagent grade sesame oil. The solution was injected subcutaneously at 0.5 ml/kg for a final dose of 50 µg/kg. Similar treatment was reported to result in plasma levels comparable to estradiol peak during the proestrus phase of the estrous cycle (Larson, Roth, Anker, & Carroll, 2005). Sesame oil (OIL; 0.5 ml/kg) was used in vehicle-treated rats.

Experiment 3. Progesterone was dissolved in reagent grade sesame oil. The solution was injected subcutaneously at 1 ml/kg for a final dose of 2 mg/kg (Feltenstein et al., 2009). Sesame oil (1 ml/ kg) was used in vehicle-treated rats.

2.3.5 Apparatus

Rats were housed individually in operant-conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0× 29.0×25.5 cm) enclosed in sound-attenuating wooden compartments equipped with a fan. Each chamber was fitted with two retractable levers (Coulbourn Instruments), mounted 9 cm above the floor of the right sidewall. Responses on the active lever activated the infusion pump and a cue-light/tone (Coulbourn Instruments, Sonalert, 2.9 KHz) complex located above the lever. Responses on the inactive lever had no programmable consequences. Rats were attached to the infusion pump via a liquid swivel (Lomir Biomedical Inc., Quebec, Canada) and Tygon tubing shielded with a metal spring.

2.3.6 Procedure

Self-administration.

Following a 24-h habituation period in the operant conditioning chambers, rats were trained to self-administer heroin daily, in three 3-h sessions separated by 3-h intervals for 10–12 days. The first daily session began shortly after the onset of the dark phase with the extension of the active and inactive levers into the operant-conditioning chamber, illumination of a house-light and activation of the cue-light/tone complex for 30 s. Responses on the active lever, which were reinforced according to a fixed ratio-1 schedule (FR-1), resulted in activation of the heroin 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.

Drug withdrawal

Experiment 1. Following self-administration training, rats were individually housed in the ACF and given unrestricted access to food and water for 1 drug-washout day. Rats then underwent

bilateral ovariectomy or sham surgery and hormone capsule implantation as described above, and allowed 2 days of recovery. Next, rats were divided into six groups: sated-SHAM-CH, sated-OVX-CH, sated-OVX-E2, FDR-SHAM-CH, FDR-OVX- CH, and FDR-OVX-E2, that were matched according to body weight, number of infusions taken, and active lever responses made over the last 5 days of training. Following recovery, FDR rats had their food restricted to approximately 12 g of rat chow delivered at 1330 h. The amount of food was adjusted through 14 days of food restriction to maintain the FDR rats' body weight (BW) to approximately 75–80 % of the sated rats and 90 % of their baseline BW.

Experiment 2. As in exp. 1, rats were allowed 1 drug-washout day prior to undergoing bilateral ovariectomy surgery. Following recovery, rats were divided into four groups: sated-OIL, sated-EB, FDR-OIL, and FDR-EB that were matched as described above. Rats were treated as in exp. 1 with the exception of EB or OIL injections, which were administered daily (0900 h) on the last 8 days of the withdrawal period.

Experiment 3. Following training, rats were allowed 1 drug-washout day and then divided into four groups: sated-OIL, sated-P, FDR-OIL, and FDR-P that were matched as described above.

Drug-seeking test

Test sessions in all experiments were initiated shortly after the onset of the dark phase.

Experiment 1: The effect of chronic treatment with low dose E2 on the food-restriction-induced augmentation of heroin seeking in female rats under withdrawal

On food restriction day 14, rats were returned to the operant conditioning chambers and attached to the metal spring. The drug-seeking test consisted of a 3-h session during which active lever responses had the same consequences as in training, however, heroin was not available.

Experiment 2: The effect of chronic treatment with high dose EB on the food-restriction induced augmentation of heroin seeking in female rats under withdrawal

The testing procedure was similar to exp. 1, with the exception that rats were administered the last daily subcutaneous injections of EB or OIL 2 h before the test session.

Experiment 3: The effect of acute treatment with progesterone on food-restriction induced augmentation of heroin seeking in female rats under withdrawal

The testing procedure was similar to exp. 1, however, rats were administered a subcutaneous injection of progesterone or OIL 20 and 2 h before the test session.

Plasma estradiol/progesterone determination

For exp. 2 and 3, for which we did not have any data on the expected levels of hormones in plasma following treatment, tail blood was collected immediately following the drug- seeking test (12 h30), and plasma was separated by centrifugation at 10,000 rpm for 10 min. Samples were stored at −80 °C. Plasma samples were analyzed for EB levels (exp. 2; sensitivity 9.714 pg/ml) or progesterone (exp. 3; sensitivity 45 pg/ml) using specific enzyme-linked immunosorbent assay (ELISA) kits (IBL—America, Minneapolis, MN).

2.3.7 Statistical analyses

All analyses were conducted using SPSS software (IBM, SPSS Statistics, version 20).

Body weight on test day and number of responses on the active and inactive levers during the drug-seeking test sessions were analyzed separately using two-way ANOVAs with feeding condition (sated, FDR) and hormone condition (exp. 1: SHAM-CH, OVX-CH, OVX-E2; exp. 2: OIL, EB; exp. 3: OIL, P) as between subject factors. In all analyses, statistically significant interactions were investigated with Fisher's protected least significant difference post-hoc test and the critical threshold for statistically significant results was set at $p \leq .05$. Effect sizes for ANOVA tests are reported using η^2, and for t tests using Hedges' g.

2.4 RESULTS

Mean±SEM number of infusions and number of active and inactive lever responses made on the final day of heroin self- administration training, for each experiment, are shown in **Table 2.1**.

	Infusions	Active lever	Inactive lever
Exp. 1	44.03±3.09	141.72±19.59	17.39±3.30
Exp. 2	49.79±4.80	173.79±29.51	15.69±3.33
Exp. 3	52.39±3.08	174.79±21.63	15.23±2.69

Table 2.1. Mean ± SEM number of infusions taken and the number of active and inactive lever responses made on the last training day (over 9 h) in each experiment

Experiment 1: The effect of chronic treatment with low-dose E2 on the food-restriction-induced augmentation of heroin seeking in female rats under withdrawal

Two rats were excluded due to failure to train, and two were excluded due to high-response rates on the inactive lever. Consequently, the analyses were conducted on 36 rats in six experimental groups: sated-SHAM-CH (n = 4), sated-OVX- CH (n = 6), sated-OVX-E2 (n = 8), FDR-SHAM-CH (n = 5), FDR-OVX-CH (n = 6), and FDR-OVX-E2 (n = 7). On test day, the average body weight (in grams) of the food-restricted rats (n = *18*; 224.40±4.70) was statistically significantly lower than that of the sated rats (n = *18*; 303.40±5.17; feeding condition: $F_{(1, 30)} = 157.13$, $p < .001$, $\eta^2 = .79$). Additionally, lower average body weights were observed in the OVX-E2 group (n = 15, 255.80±11.19) compared to the OVX-CH (n = *12*, 269.25±15.42) and SHAM-CH groups (n = *9*, 270.22±12.76). A statistically significant main effect of hormone condition ($F_{(2, 30)} = 4.19$, $p = .025$, $\eta^2 = .04$) was observed, however, further post-hoc analysis did not reveal any statistically significant differences between hormone groups. No statistically significant effect for the feeding condition X hormone condition interaction was found ($F_{(2, 30)} = 1.95$, $p = .159$, $\eta^2 = .02$; see **Fig. 2.1** for body weight changes over training and withdrawal periods).

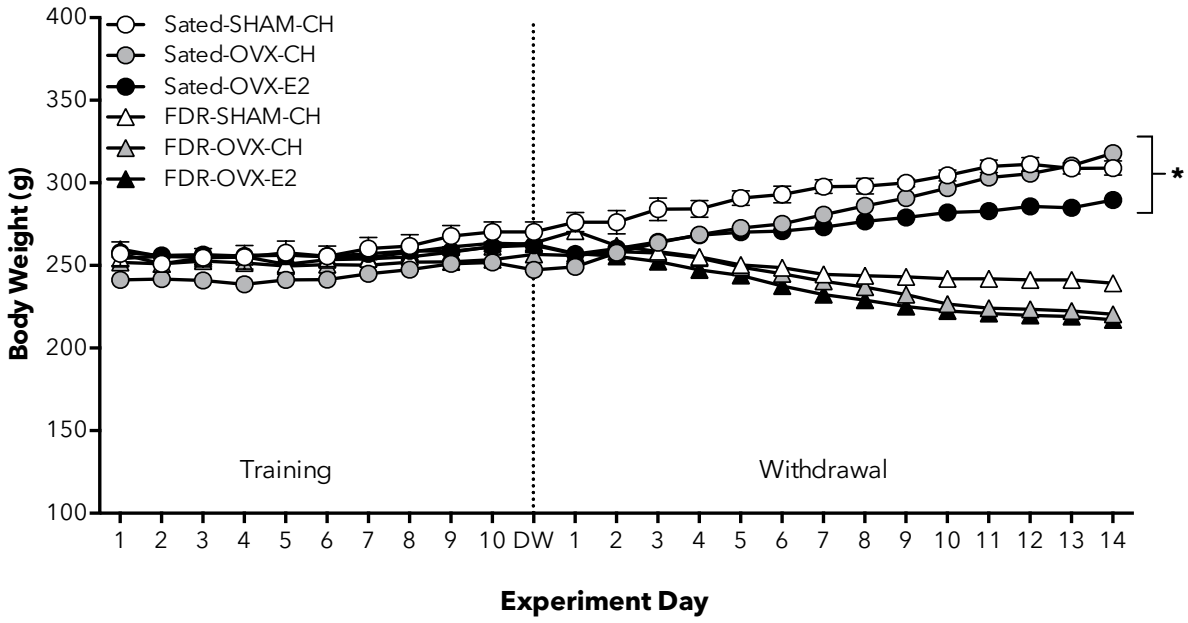

Figure 2.1. Body weights during heroin self-administration training and withdrawal; Experiment 1. Mean (±SEM) body weights for rats in the food- restricted (FDR; *ns:* SHAM-CH = 5, OVX-CH = 6, OVX-E2 = 7) and sated (*ns:* SHAM-CH = 4, OVX-CH = 6, OVX-E2 = 8) groups. SHAM intact females, OVX ovariectomized females, CH cholesterol, E2 17β-estradiol, DW drug-washout day. *****Statistically significant difference from the food-restricted rats on day 14 of food restriction, $p < .001$
Note: Figure adapted from Sedki et. al., 2015.

On test day, food-restricted rats demonstrated augmented active lever responding (348.17±26.20), compared to their sated counterparts (245.17±27.98; feeding condition: $F_{(1, 30)} = 10.27$, $p = .003$, $\eta^2 = 0.21$; **Fig. 2.2A**). Furthermore, a statistically significant feeding condition X hormone condition interaction was observed ($F_{(2, 30)} = 3.73$, $p = .036$, $\eta^2 = 0.16$). Subsequent post-hoc analyses revealed enhanced heroin seeking in the FDR-SHAM-CH rats compared to the sated- SHAM-CH rats ($p = .021$, $g = 2.08$). Similarly, the FDR- OVX-CH group demonstrated greater levels of heroin seeking compared to the sated-OVX-CH group ($p = .004$, $g = 1.74$). Estradiol replacement in the food-restricted OVX rats reduced the augmentation in heroin seeking compared to cholesterol replacement, while the same treatment somewhat increased heroin seeking in the sated rats. Consequently, there was no statistically significant difference

between the FDR-OVX-E2 and sated-OVX-E2 groups (p = .806, g = 0.10). No statistically significant main effect of hormone condition on active lever responses was observed ($F_{(2, 30)}$ = 0.10, p = .905, η^2 = 0.004). There were no statistically significant main effects (feeding condition: $F_{(1, 30)}$ = 1.21, p = .281, η^2 = 0.04, hormone condition: $F_{(1, 30)}$ = 0.60, p = .556, η^2 = 0.04) or an interaction effect ($F_{(2, 30)}$ = 0.79, p = .464, η^2 = 0.05) on inactive lever responses (**Fig. 2.2B**).

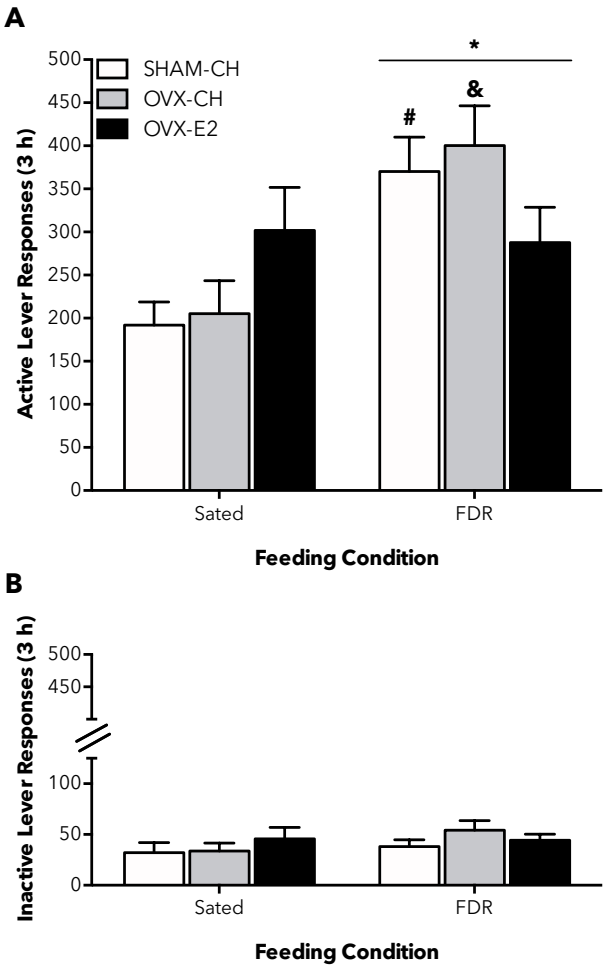

Figure 2.2. The effect of treatment with chronic low dose 17β-estradiol (E2) on the food-restriction-induced augmentation of heroin seeking in female rats under withdrawal; Experiment 1. Data shown represent mean (+SEM) numbers of active (**A**) and inactive (**B**) lever responses made on test day by rats in the food-restricted (FDR; *ns:* SHAM-CH = 5, OVX-CH = 6, OVX-E2 = 7) and sated (*ns:* SHAM-CH = 4, OVX-CH = 6, OVX-E2 = 8) groups. *****Statistically significant difference from the sated groups, p = .003. **#,&**Statistically significant difference from the same hormonal treatment group under sated condition (p = .021, p = .004, respectively)
Note: Figure adapted from Sedki et. al., 2015.

Experiment 2: The effect of chronic treatment with high-dose EB on the food-restriction-induced augmentation of heroin seeking in female rats under withdrawal

One rat was removed from statistical analyses due to failure to train. Therefore, final analysis included 29 rats in four experimental conditions: sated-OIL (n = 7), sated-OIL+EB (n = 7), FDR-

OIL (n = 7), and FDR-EB (n = 8). On test day, rats in the sated condition (n = 14, 344.21±9.34 g) weighed more than those in the FDR condition (n = 15, 256.13±5.26 g; feeding condition: $F_{(1, 25)}$ = 147.51, p < .001, η^2 = .72). Treatment with EB resulted in an overall lower body weight (hormone condition: $F_{(1, 25)}$ = 18.29, p < .001, η^2 = .09). This effect was driven by the lower body weight in the sated-EB group compared to the sated-OIL group (p < .0001, g = 3.15), and the lack of EB effect on body weight in the food-restricted groups (feeding condition X hormone condition: $F_{(1, 25)}$ = 14.96, p < .001, η^2 = .07; **Fig. 2.3**).

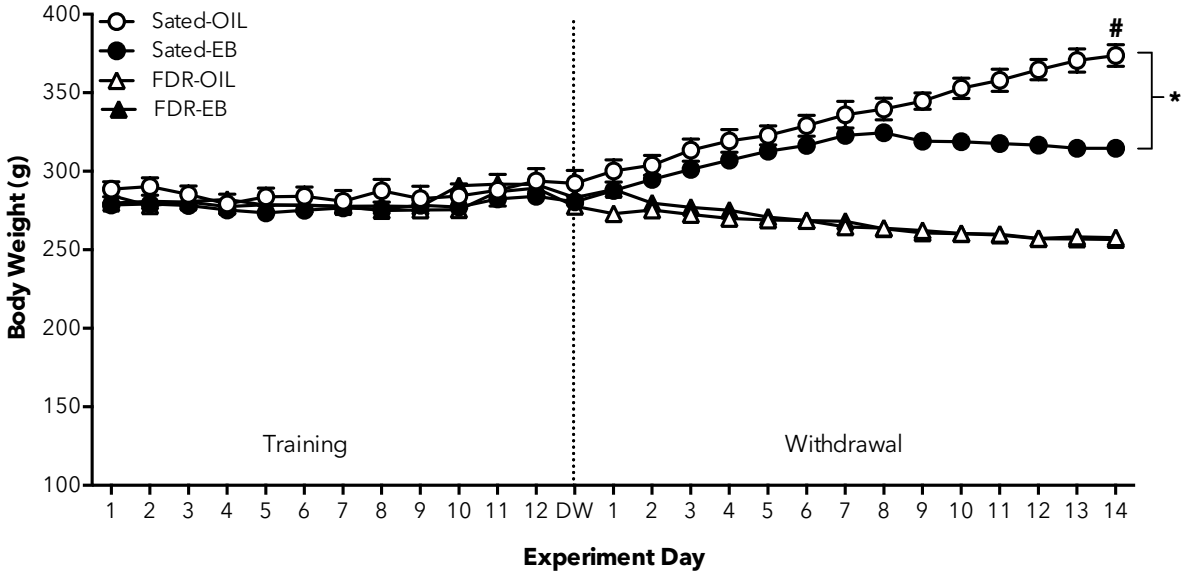

Figure 2.3. Body weights during heroin self-administration training and withdrawal; experiment 2. Mean (±SEM) body weights across experiment 2 in the food-restricted (FDR; *ns:* OIL = 7, EB = 8) and sated (*ns:* OIL = 7, EB = 7) groups. EB 17β-estradiol 3-benzoate, DW drug-washout day. *****Statistically significant difference from the food-restricted rats on day 14 of food restriction, p < .0001. **#**Statistically significant difference from the sated-EB group on Day 14 of food restriction, p < .0001
Note: Figure adapted from Sedki et. al., 2015.

On test day, rats subjected to a mild chronic food restriction demonstrated augmented active lever responding (303.80± 32.73), compared to their sated counterparts (224.07 ± 19.43; feeding condition: $F_{(1, 25)}$ = 5.55, p = .027, η^2 = .37; **Fig. 2.4A**). Chronic administration of high-dose EB attenuated active lever responding (n = 15, 228.07±13.80) compared to the OIL group

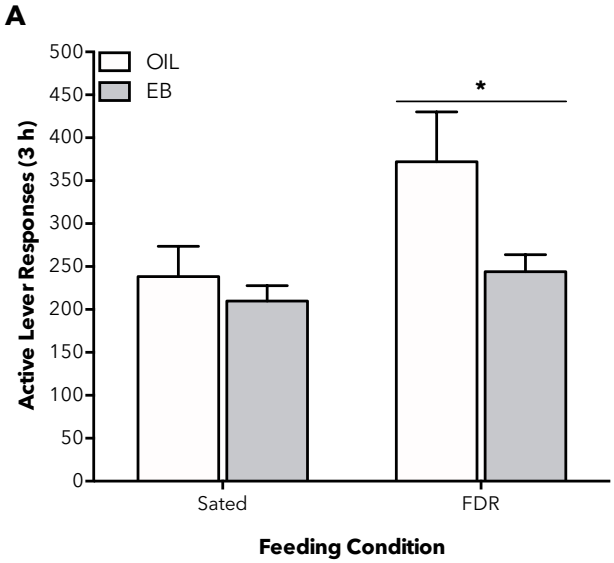

Figure 2.4. The effect of treatment with chronic high dose 17β-estradiol 3-benzoate (EB; 50.0 ug/kg) on the food-restriction-induced augmentation of heroin seeking in female rats under withdrawal; experiment 2. Mean (+SEM) numbers of active (**A**) and inactive (**B**) lever responses made on test day by rats in the FDR (*ns:* OIL = 7, EB = 8) and sated (*ns:* OIL = 7, EB = 7) groups. *****Statistically significant difference from the sated groups, $p = .027$
Note: Figure adapted from Sedki et. al., 2015.

(n = 14, 305.21±37.55; hormone condition: $F_{(1,25)} = 4.82$, $p = .038$, $\eta^2 = .33$). The statistically significant effect of EB treatment seemed to be driven mostly by the inhibition of active lever responses in the FDR group ($g = 1.08$), although no statistically significant effect of feeding condition X hormone condition interaction was observed ($F_{(1, 25)} = 1.93$, $p = .177$, $\eta^2 = .13$). No statistically significant main effects (feeding condition: $F_{(1, 25)} = 0.17$, $p = .686$, $\eta^2 = 0.01$, hormone condition: $F_{(1, 25)} = 0.13$, $p = .721$, $\eta2 = 0.005$) or interaction ($F_{(1, 25)} = 2.06$, $p = .164$, $\eta^2 = 0.08$) were found for inactive lever responses (**Fig. 2.4B**).

Plasma levels of E2 in the ovariectomized rats were below the reported sensitivity level of the ELISA kit. Chronic treatment with EB resulted in supraphysiological levels of plasma E2,

between 262.58±45.63 pg/ml (sated group) and 379.53± 56.26 pg/ml (FDR group). The difference in plasma E2 levels between the feeding condition groups was not statistically significant (p = 0.14).

Experiment 3: The effect of acute treatment with progesterone on food-restriction-induced augmentation of heroin seeking in female rats under withdrawal

One rat was removed from analysis due to catheter leakage, and one rat from the progesterone-treated group was removed from all analyses due to an extremely high value of plasma progesterone (119 ng/ml vs. a group mean of 25.9 ng/ml). Thus, analysis included 38 rats in four experimental conditions: sated-OIL (n = 10), sated-P (n = 8), FDR-OIL (n = 11) and FDR-P (n = 9). On test day, the sated group (n = 18, 295.50±4.71 g) weighed more than the FDR group (n = 20, 248.10±2.26 g; feeding condition: $F_{(1, 34)}$ = 87.49, p < .001, η^2 = .71). No statistically significant effects for the hormone condition ($F_{(1, 34)}$ = 2.17, p = .150, η^2 = .02) or the feeding condition X hormone condition interaction ($F_{(1, 34)}$ = 0.01, p = .939, η^2 = .00005) were observed.

During the drug-seeking test, food-restricted rats responded more on the active lever (388.00 ± 35.34), compared to sated rats (221.72±19.93; feeding condition: $F_{(1, 34)}$ = 15.04, p < .001, η^2 = .31; **Fig. 2.5A)**. No statistically significant effects on active lever responding were observed for the hormone condition ($F_{(1, 34)}$ = 0.09, p = .771, η^2 = .002) or feeding condition X hormone condition interaction ($F_{(1, 34)}$ = 0.06, p = .810, η^2 = .001). There were no statistically significant main (feeding condition: $F_{(1,34)}$ = 0.0004, p = .985, η^2 = .00001, hormone condition: $F_{(1, 34)}$ = 0.86, p = .361, η^2 = .02) effects or interaction ($F_{(1, 34)}$ = 0.04, p = .839, η^2 = .001) on inactive lever responses (**Fig. 2.5B**).

Plasma samples from 5 rats were not analyzed due to difficulty in blood collection or hemolysis. Progesterone treatment resulted in a statistically significant increase in plasma progesterone (n = 16, 24.70 ± 2.59 ng/ml) compared to the oil- treated rats (n = 17, 13.0±1.80 ng/ml; hormone condition: $F_{(1, 29)}$ = 13.35, p = .001, η^2 = .31). No other statistically significant differences were found.

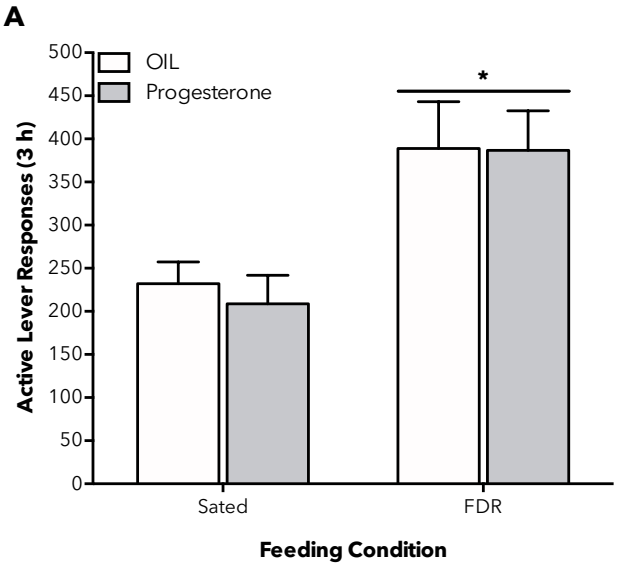

Figure 2.5. The effect of acute treatment with progesterone (P; 2.0 mg/kg) on food-restriction-induced augmentation of heroin seeking in female rats under withdrawal; experiment 3. Mean (+SEM) numbers of active (**A**) and inactive (**B**) lever responses made on test day by rats in the food restricted (FDR; *ns:* OIL = 11, OIL+P = 9) and sated (*ns:* OIL = 10, OIL+P = 8) groups. *****Statistically significant difference from the sated groups, $p < .001$.

Note: Figure adapted from Sedki et. al., 2015.

2.5 DISCUSSION

We used ovariectomy and hormone treatments to study the role of ovarian hormones in the augmentation of heroin seeking in food-restricted female rats under withdrawal. The first important finding is that, as in males, chronic mild food restriction results in the augmentation of heroin seeking following prolonged withdrawal in female rats. Second, ovariectomy had no statistically significant effect on heroin seeking in the sated or food-restricted rats. However, chronic pre-treatment with estradiol, at both doses tested, blocked food-restriction- induced augmentation of heroin seeking in the ovariectomized rats. Finally, progesterone administration did not reduce hero- in seeking in chronically food-restricted or sated rats under withdrawal conditions.

Recent work in our laboratory has demonstrated a robust augmentation in heroin seeking following prolonged food restriction in male rats under withdrawal (D'Cunha et al., 2013). In the current study, we found that intact females that did not receive hormone treatment exhibited a robust augmentation in heroin seeking. These findings are in agreement with considerable evidence that supports a modulatory role for food restriction on drug-related behaviours in humans (Cheskin et al., 2005; Hall et al., 1992; Krahn et al., 1992) and in laboratory animals (Carr, 2007; Carroll, 1985; Stuber et al., 2002), where food deficiency drastically increases drug taking and the reinforcing properties of psychoactive drugs.

While sex differences in ethanol and stimulant drugs seeking and taking have been established (Carroll & Anker, 2010), similar reports with opiates are sparse and inconsistent. Stewart and colleagues (Stewart et al., 1996) failed to identify any meaningful differences between sexes in the rates of heroin self-administration or the reinforcing effects of heroin. In contrast, it was reported that female rats self-administered more heroin and morphine than males (Cicero et al., 2003), and acquired heroin self-administration more rapidly than males (Lynch & Carroll, 1999). Here, we did not directly compare drug seeking in male and female rats, and thus conclusions regarding sex differences or similarities should be considered with caution; however, the augmentation of heroin seeking in the food-restricted female rats under withdrawal was similar to the one previously observed in male rats (D'Cunha et al., 2013; Sedki, Abbas, Angelis, Martin, et al., 2013a). Our data are in agreement with, and extend, the results reported by Carroll and colleagues (2001) who found no sex differences in acute food-restriction-induced enhancement of heroin intake.

Contrary to our expectations, chronic low or high dose estradiol replacement did not enhance, but instead attenuated heroin seeking following prolonged food restriction. In addition, both the low and high doses of estradiol replacement resulted in similar attenuation of heroin seeking in the food- restricted rats to the level observed in the sated rats, but not below. This lack of dose effect suggests that the effect of estradiol was specific to food-restriction-induced augmentation of heroin seeking, and that even a relatively low circulating dose of the hormone is sufficient to prevent this augmentation. The data presented here are inconsistent with

previous reports of reduced cocaine seeking following ovariectomy, and a reversal of this attenuation with estradiol replacement (Larson et al., 2005). Our findings also contradict a more recent report that rats in the proestrus phase (high circulating estradiol) of the estrous cycle demonstrate the greatest levels of cue and stress-induced reinstatement of cocaine seeking (Feltenstein, Henderson, & See, 2011). One possible explanation for our finding is that the attenuation of heroin seeking observed here resulted from estradiol's anxiolytic properties. Treatment with estradiol was shown to have anxiolytic effects in the open field (i.e., more center entries) and elevated plus maze (more time in open arms) tests (Bowman, Ferguson, & Luine, 2002; Walf & Frye, 2005). Importantly, estradiol treatment also moderated the anxiogenic effect of restraint stress in these tests (Bowman et al., 2002; Walf & Frye, 2005) In humans, food restriction causes physiological and negative affective states that drive physiological stress responses, such as an increase in cortisol levels (Tomiyama et al., 2010). In addition, pre-clinical data indicate that as with other stressors, food deficiency can in- crease corticosterone release in rodents (Dallman et al., 1999; Marinelli, Le Moal, & Piazza, 1996). Furthermore, adaptations in stress- related mechanisms that are implicated in the resumption of drug seeking, such as the extrahypothalamic corticotropin releasing factor (CRF) system, were suggested to underlie the augmenting effects of dietary restriction on drug reward and drug seeking (Shalev, Finnie, Quinn, Tobin, & Wahi, 2006). This explanation, however, seems unlikely. Anker and Carroll (2010) reported that female rats are more sensitive than males to stress-induced reinstatement of cocaine seeking. These results suggest that estradiol in cycling female rats does not protect from, but rather augments, the effects of stress on drug seeking. Finally, recent findings from our laboratory suggest that acute, stress- related activation of the CRF and hypothalamic-pituitary- adrenal (HPA) axis systems is not critical for the expression of augmented heroin seeking in chronically food-restricted male rats (Sedki, Abbas, Angelis, Martin, et al., 2013a).

An alternative explanation for the attenuated food-restriction-induced augmentation of heroin seeking in the estradiol-treated rats could be the anorexigenic properties of the hormone. Ovariectomy promotes increased eating and weight gain, while administrations of estradiol to ovariectomized rats increases satiety resulting in reduced eating and rate of weight gain (Wade,

1975). The anorexigenic effects of estradiol were clearly observed in this study, for example, in the sated-EB group (Exp. 2) that showed a statistically significantly slower gain in body weight over the withdrawal period (Fig. 3). Importantly, we have reported that elimination of the hunger state with acute re-feeding reverses the effect of chronic food restriction on heroin seeking in male rats (D'Cunha et al., 2013). The small increase in heroin seeking observed in the sated-OVX-E2 group (Exp. 1) could be interpreted as an estradiol-induced augmentation of drug seeking, albeit expressed only in sated rats. However, the effect was not replicated when higher plasma estradiol levels were induced in the sated rats in Exp. 2.

The present data partly contradict previous findings which purport an attenuating role for progesterone on cocaine seeking (Anker, Larson, Gliddon, & Carroll, 2007; Feltenstein et al., 2009). Progesterone was shown to attenuate cocaine priming-induced reinstatement of extinguished cocaine seeking in intact female rats, and progesterone pre-treatment reversed the augmentation of cocaine seeking in estradiol-treated ovariectomized rats (Anker et al., 2007). Feltenstein and colleagues (2009) report- ed that in cycling female rats, cocaine priming-induced rein- statement of extinguished cocaine seeking was increased in females in the estrus phase compared to rats in the other phases of the estrous cycle. Progesterone treatment before the reinstatement test selectively blocked this augmentation, but was ineffective for rats not in estrus. It is important to note that in both the above studies, progesterone treatment did not block the priming-induced reinstatement effect itself. However, more recently Anker and Carroll (2010) reported that the progesterone metabolite, allopregnanolone, blocked reinstatement of extinguished cocaine seeking induced by the pharmacological stressor, yohimbine, in female rats.

There are two possible explanations for the discrepancy between our findings and the ones cited above. First, as mentioned before, ours is one of the few studies that assessed the effects of ovarian hormones on heroin seeking. Most previous reports, including Anker et al. (2007) and Feltenstein et al. (2009), utilized cocaine-trained rats. There are important differences in the long-lasting brain adaptations following prolonged exposure to heroin or cocaine, as well as in the brain circuits involved in the reinstatement of heroin and cocaine seeking (Badiani et

al. 2011; Rogers et al. 2008). Second, there are procedural differences between these previous studies and the current one. While drug seeking is most often assessed using the reinstatement procedure (i.e., following an extinction training), we tested the effects of ovarian hormones on heroin seeking following an extended withdrawal period (see D'Cunha et al., 2013; Fuchs et al., 2006; 2008), for a discussion on the implications of such procedural differences). In agreement with the present data, Feltenstein and colleagues (2009) reported that progesterone treatment had no effect on cocaine seeking during withdrawal in any of the estrous phases, albeit in early withdrawal (the first day of drug-seeking extinction that followed cocaine self- administration training).

In conclusion, our findings suggest that manipulations of plasma levels of ovarian hormones have little effect on the expression of cue-induced heroin seeking following a prolonged withdrawal period, or on the augmentation of this effect in food-restricted rats. However, and contrary to our expectation, treatment with estradiol, but not progesterone, can modulate heroin seeking in food-restricted rats. There is considerable evidence, from both clinical and pre-clinical studies, that fluctuations in circulating ovarian hormones can modulate some motivational effects of drugs of abuse. Our findings suggest that further research is needed to fully under- stand the potential of hormonal manipulations in the treatment of drug seeking in women under different environmental challenges and with various drug types.

Chapter 3: Changes in AMPA GluA1 receptor subunit associated with augmented heroin seeking in chronically food-restricted rats under withdrawal

3.1 ABSTRACT

Rationale. Following prolonged withdrawal, food-restricted rats demonstrate augmented heroin seeking upon re-exposure to heroin cues. Neuroadaptations in the glutamate system are thought to underlie the vulnerability to return to heroin seeking following long-term withdrawal. The specific mechanism of this vulnerability remains unclear.

Objectives. We tested the effects of caloric restriction on total and phosphorylated GluA1 receptor protein subunit levels in drug-naïve and heroin-seeking rats.

Methods. Male rats were trained to self-administer heroin in operant conditioning chambers for 10 days. Next, rats were moved to the animal facility and remained sated or underwent chronic food restriction for 14 days. On day 14, rats were sacrificed before or after a 1 h heroin-seeking test (extinction conditions). Next, Western blots identified relative total GluA1-R and phosphorylated GluA1-R (p-Ser845-GluA1) protein levels in the NAc and mPFC. Drug naïve, sated and food-restricted rats were used as controls.

Results. Following withdrawal, re-exposure to heroin cues increased p-Ser845-GluA1 receptor protein levels in the NAc shell compared to heroin-trained rats sacrificed before the heroin-seeking test. Reductions in NAc shell total GluA1 and p-Ser845-GluA1 receptor protein levels were detected in heroin-trained rats compared to drug-naïve rats. Adaptations in the GluA1 receptor were not observed due to dietary restrictions.

Conclusions. Following prolonged withdrawal, exposure to heroin cues may reverse the reduction in GluA1 receptor protein phosphorylation observed in heroin-trained rats. However, the role of AMPARs in calorically restricted rats remains unclear. As the present study did not dissociate membrane-bound and cytoplasmic receptors, these findings may be a limitation of the molecular procedures employed, and further work is needed to clarify the role of the GluA1 receptor in chronically food-restricted, heroin-seeking rats.

Keywords Food restriction · Heroin seeking · Glutamate · AMPARs · GluA1 · Nucleus accumbens

3.2 INTRODUCTION

The reinforcing properties of a wide variety of abused drugs are modulated by disturbances in caloric homeostasis (Campbell & Carroll, 2001; Carr, 2002; Carroll et al., 2001; Carroll & Meisch, 1981; Cheskin et al., 2005; D'Cunha et al., 2013; Zacny & de Wit, 1991). For example, chronic exposure to food restriction increases psychostimulant and opioid self-administration compared to sated rats (Carroll et al., 2001; Carroll & Meisch, 1981). These drug enhancing effects of caloric restriction extend beyond drug taking, as we have reported that cue-induced heroin seeking is also augmented in food-restricted rats following prolonged withdrawal (D'Cunha et al., 2013; Sedki, Abbas, Angelis, Martin, et al., 2013a; Sedki, D'Cunha, & Shalev, 2013b). Unfortunately, little is known about the underlying mechanisms that prompt the augmentation of heroin-seeking behaviour.

Mechanistic studies indicate that drugs of abuse can persistently alter critical neuronal circuitry, driving the vulnerability to relapse long after the cessation of drug use (Shen et al., 2011; Thomas et al., 2001). Of great interest are the neuroadaptations in the glutamatergic system resulting from drug exposure, which are thought to be strong contributors to the persistence of drug relapse (Kalivas, 2009; Kalivas et al., 2009). Accordingly, pre-clinical work has revealed intra-NAc increases of the ionotropic glutamate receptor subtype AMPA containing the GluA1 subunit during prolonged withdrawal from cocaine (Conrad et al., 2008). Furthermore, injections of AMPA into the NAc was shown to reinstate cocaine-seeking behaviours (Cornish & Kalivas, 2000). Systemic and intra-NAc injections of the AMPAR antagonist CNQX were also reported to reduce cue- and context-induced reinstatement of cocaine seeking, respectively (Bäckström & Hyytiä, 2006). Thus, a clear pattern emerges where manipulations aimed at the blockage of AMPARs decrease, while those meant to excite AMPARs increase, drug seeking.

Interestingly, a narrative exists in which abused drugs and restricted diets can independently increase AMPAR subunit levels, but the combined impact of both can further enhance this augmentation. For example, while chronically food-restricted drug-naïve rats exhibit elevated

levels of GluA1 and GluA2 in the NAc, these effects are further augmented following an amphetamine challenge (Peng et al., 2014). More so, enhanced phosphorylation of GluA1 at the ser845 residue site in the NAc has also been observed in chronically food-restricted, amphetamine-treated rats, compared to sated rats (Peng et al., 2015; 2014). In line with the comprehensive review by Carr (2016), a strong case is made for the modulatory impact of dietary restriction on ser845 phosphorylation and trafficking of GluA1 receptors to the cell membrane in rats treated with psychostimulants.

The role of the GluA1 receptor in opiate-seeking behaviour, however, remains unclear. While there is strong evidence that increases in synaptic strength of glutamatergic projections from the mPFC to the NAc core are required for the expression of heroin-primed reinstatement (Shen et al., 2011), evidence on the direct impact on the GluA1 receptor subunit protein remains limited. To our knowledge, only one study has investigated the effects of AMPAR subunit changes following exposure to heroin-conditioned cues in rats under withdrawal, which resulted in a downregulation of mPFC GluA2, and no change in the GluA1 AMPAR subunits (Van den Oever et al., 2008). Yet, Vanden Oever and colleagues (2008) did not evaluate the impact of caloric restrictions on heroin cues. In fact, as elevated NAc GluA1 receptor levels are observed in food-restricted rats (Peng et al., 2015), it remains difficult to infer the influence that caloric restriction may have in rats exposed to heroin-conditioned cues.

Therefore, the purpose of the present study was to explore the impact of caloric restriction on total and phosphorylated GluA1 receptor protein subunit levels in heroin-seeking rats. Of particular interest were the changes in receptor proteins in response to caloric restriction, and the influence of exposure to heroin-associated cues on such protein level changes. Given the aforementioned reports and recent findings that food restriction can increase the synaptic incorporation of phosphorylated and total GluA1 receptor subunit protein levels in the NAc (Ouyang et al., 2017), we expected that our food restriction procedure would generate increases in GluA1 levels in drug-naïve and drug-trained rats. We also note strong evidence that DA-D1-R activation is necessary for the expression of psychostimulant and opiate-seeking

behaviours (Alleweireldt, Weber, Kirschner, Bullock, & Neisewander, 2002; Bossert, Poles, Wihbey, Koya, & Shaham, 2007; D'Cunha et al., 2017). In agreement with these findings, our laboratory has demonstrated that augmented heroin seeking in chronically food-restricted rats can be selectively inhibited following administration of a DA-D1-R antagonist (SCH39166) in the NAc core (D'Cunha et al., 2017). One downstream effect of DA-D1-R activation includes increases in the trafficking, phosphorylation, and insertion of the iGluR subtype AMPA into the cell membrane (Barry & Ziff, 2002; Gao & Wolf, 2007). Therefore, it is conceivable that exposure to a heroin-seeking test may increase the levels of the GluA1 subunit beyond those expected in response to food restriction alone, in heroin-trained but not in not-tested control rats.

3.3 METHODS

3.3.1 Subjects

A total of 60 male, Long Evans rats (Charles River, St Constant, Quebec, Canada or New Jersey, USA; 275-300 g) were used. Before surgery, rats were pair-housed and handled daily for one week in the ACF under 12 h reverse light/dark conditions (lights OFF at 0930). Following IV catheterization, rats were single-housed in plastic shoebox cages and allowed two days of recovery. Rats were then transferred to operant conditioning chambers for heroin self-administration. Following self-administration training, rats were returned to the ACF and single-housed in shoebox cages for the drug-washout and withdrawal periods. At the end of the withdrawal period, rats were returned to the operant conditioning chambers for testing and then sacrificed. Except for the withdrawal and testing phases, all rats were allowed 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.

3.3.2 Surgical Procedures

All rats were implanted with IV silastic catheters (Dow Corning, Midland, MI, USA) under xylazine/ketamine (10.0+100.0 mg/kg; ip) anesthesia, as described previously (Sedki, D'Cunha,

& Shalev, 2013b). Following surgery, rats were injected with saline (0.9%) for hydration, penicillin (450 000 IU/rat; s.c.) to prevent infection and the analgesic buprenorphine (10.0 µg/kg; s.c.; Schering-Plough Ltd., Welwy Garden City Hertfordshire, UK) to reduce pain. Catheters were flushed daily throughout the self-administration phase, with heparin/gentamicin (7.5 UI + 12.0 mg/rat) to prevent blockage and infection.

3.3.3 Apparatus

During self-administration and testing, rats were individually housed in operant conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 X 29.0 X 25.5 cm) enclosed in a sound attenuating wooden cubicle equipped with a fan (noise rating of 45 dBA; Newark, Canada). The front and back walls of the chambers were built using polycarbonate plexiglass, while the ceiling and side walls were composed of stainless steel. Each chamber was fitted with two retractable levers mounted 9 cm above the floor of the right sidewall. All rats were attached to an infusion pump (3.33 rpm), located above the chamber via a liquid swivel (Lomir Biomedical Inc., Notre-Dame-de-L'île-Perrot, QC, Canada) and Tygon tubing shielded with a metal spring. A red house light was installed at the top center of the left sidewall, and a cue-light was set above each lever. A tone emitter (Coulbourn Instruments; Sonalert, 2.9 KHz) was located directly above the active (heroin-paired) lever.

3.3.4 Drug

Heroin HCl (a generous contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in sterile saline (Heroin: 5.0 mg/kg) and then further diluted with saline to yield a dose of 0.1 mg/kg/infusion adjusted for each rat according to BW.

3.3.5 Procedure

Self-Administration (10 days). Following recovery, rats were allowed a 24 h habituation period to the operant conditioning chambers. Next, rats were trained to self-administer heroin in daily, three 3 h sessions separated by 3 h intervals for 10 days. The first session began within 30 min after the onset of the dark phase with the extension of the levers into

the operant conditioning chamber, and activation of a cue-light/tone complex above the active lever and red house light for 20 s. Responses on the active lever, programmed with a fixed interval 20 s schedule of reinforcement (FI-20 s), resulted in activation of the drug pump (5 s; 0.13 ml/infusion). During the 20 s interval, the house light was turned off, the cue light/tone complex was activated, and active lever responses were recorded but not reinforced. Responses on the alternative (non-paired) lever were recorded but had no programmed consequences. Following each 3 h self-administration session, the active lever was retracted whereas the alternative lever was only retracted 1 h prior to the first session on the next day.

Withdrawal (15 days). Following self-administration training, rats were individually housed in the ACF, and allowed unrestricted access to food and water for one drug-washout day. The next day, rats were separated into two groups: Sated, or food-restricted (FDR). Groups were matched according to BW, number of infusions, and active lever responses across the last 5 days of training. Following the washout day, on the first day of the withdrawal phase, rats in the FDR group had their food removed and were fed ~ 15 g of chow at 1330 h. The amount of food was adjusted throughout the 14 days of food restriction to maintain the food-restricted rats' BW at ~ 90% of their W2 BW.

Test (1 day). On W15, rats (Tested group) were returned to the operant conditioning chambers at 0930 h and attached to the metal spring. The heroin-seeking test consisted of one 1 h session during which active lever responses held the same consequences as in training, with the exception of drug availability. Rats were euthanized immediately after the test. A second group of rats (Not-Tested group) underwent identical training and withdrawal treatment, but were not tested, and instead euthanized between 0930-1030 h on the 15th day of withdrawal. In addition, a third group of rats that did not undergo surgery or self-administration training (naïve group) was handled and weighed daily for 11 days, exposed to 14 days of unrestricted or restricted access to chow (as described above), and then euthanized on day 14 between 0930 h and 1030 h. See **Figure 3.1A** for a summary of the experimental procedure.

Tissue collection.

Following euthanasia (decapitation after a brief exposure to CO_2), brains were quickly extracted post-test, frozen in -80°C isopentane (2-methylbutane, Fisher Scientific Canada), and stored at -80°C. Next, on ice, 0.3 mm bilateral tissue punches of the NAc (Paxinos & Watson, 2005) and prefrontal cortex (Paxinos & Watson, 2005) were obtained from 200-400 µm coronal sections sliced at -26°C on a cryostat.

3.3.6 Western Blot

Sample preparation. Tissue punches were sonicated (three-5 s cycles) in a volume of 40 µl lysis buffer solution (77% ml ddH_2O, 1% 1M Tris-HCl, pH = 6.8, 2 ml 2% SDS, 1% phosphatase inhibitor cocktails 2 and 3, and 1 tablet protease inhibitor cocktail), and then frozen in liquid nitrogen. Next, tissue punches were thawed on a heat block (37°C) three times before undergoing centrifugation (30 min; 10,000 g; 4°C). Following centrifugation, supernatant was collected and used for western blot assays. The protein concentrations of all samples were determined using the bichinconinic acid assay (ThermoFisher Scientific).

Western blot assay. Sample volumes containing 10 µg of protein were diluted in loading buffer (9% glycerol, 5% 1M Tris-HCl, pH = 6.8, 2% SDS, 2% 2-Mercaptoethanol, 0.004% bromophenol blue) and ddH_2O to a final volume of 25 µl. Each sample was centrifuged and boiled for 5 min at 95°C, before undergoing SDS-polyacrylamide gel (46% ddH_2O, 26% 1.5M Tris-HCl, 8% acrylamide, pH = 8.8, 0.1% sodium dodecyl sulfate, 0.1% ammonium persulfate, 0.1% N,N,N′,N′-Tetramethylethylenediamine) electrophoresis for 1h45 at 120 V. Proteins were transferred electrophoretically to nitrocellulose membranes (0.2 µm; Bio-Rad) at 100 V for 1 h. The saturation and blotting of membranes were performed at room temperature, using the

		Sated			FDR		
		Naïve	**Not-Tested**	**Tested**	**Naïve**	**Not-Tested**	**Tested**
GluA1	**NAc Shell**	*n* = 8	*n* = 4	*n* = 6	*n* = 8	*n* = 5	*n* = 6
	NAc Core	*n* = 4	*n* = 3	*n* = 2	*n* = 4	*n* = 3	*n* = 3
	Prelimbic Cortex	*n* = 3	*n* = 4	*n* = 4	*n* = 4	*n* = 4	*n* = 4
	Infralimbic Cortex	*n* = 3	*n* = 4	*n* = 4	*n* = 4	*n* = 4	*n* = 3
p-Ser845 - GluA1	**NAc Shell**	*n* = 8	*n* = 4	*n* = 6	*n* = 8	*n* = 6	*n* = 5
	NAc Core	*n* = 10	*n* = 5	*n* = 6	*n* = 9	*n* = 7	*n* = 8
	Prelimbic Cortex	*n* = 3	*n* = 3	*n* = 2	*n* = 4	*n* = 3	*n* = 3
	Infralimbic Cortex	*n* = 4	*n* = 3	*n* = 4	*n* = 4	*n* = 5	*n* = 4

Table 2. Number of GluA1 and p-Ser845-GluA1 samples (*n*) assayed in the NAc shell, NAc core, prelimbic cortex, and infralimbic cortex, across feeding (sated, food-restricted) and testing (naïve, not-tested, tested) conditions.

SNAP ID 2.0 protein detection system (EMD Millipore). Membranes were washed 1 X 30 s in blocking buffer: 0.1% bovine serum albumin (BSA) in tris buffered saline with 0.1% tween-20 (TBST). Next, membranes were incubated for 1 h with their primary antibody diluted in blocking buffer. The antibodies used were anti-GluR1 (1:1000 or 1:10000 dilution; EMD Millipore), ser845-anti-GluR1 (1:500; EMD Millipore), and anti-actin (1:60000; EMD Millipore). The membranes were then washed 4 X 30 s in TBST and then incubated for 10 min with horseradish peroxidase-conjugated secondary antibodies; goat anti-mouse/rabbit IgG (2:2500; EMD Millipore) and StrepTactin (0.8:5000; Bio-Rad). Finally, blots were washed 4 X 30 s in TBST and

developed for 5 min using a enhanced chemiluminescence (ECL) substrate kit (PerkinElmer, Inc.).

Imaging and analyses. Luminescence from the blots was detected directly using the Amersham Imager 600 (GE Healthcare) or indirectly using x-ray film (Denville Scientific, Inc.) followed by digital scanning. Densitometric analysis of the bands was performed using Image Studio Lite (LI-COR; version 5.2.5). Raw densities were normalized according to the following steps: ***1.*** Relative values were calculated using each sample's anti-actin optical density value and dividing by the largest anti-actin value in the Sated-Naïve group. ***2.*** Each target protein density was then divided by the calculated relative value matching its sample. ***3.*** Last, the adjusted target protein densities were normalized by dividing their calculated densities by the average of Sated-Naïve group.

3.3.7 Statistical Analyses

All analyses were conducted using Prism software (Graphpad Software, version 6.0), except for effect sizes and CIs which were calculated using R (The R Project for Statistical Computing, version 3.3.1). The critical threshold for statistically significant results was set at $p \leq 0.05$, and adjusted for multiple comparisons using the Holm-Sidak correction when necessary. All statistically significant interactions were investigated with *post-hoc* tests using the Holm-Sidak correction. All *t*-tests were followed by Cohen's *d* effect size calculations, while all analyses of variance (ANOVAs) were followed by eta-squared effect size calculations. All CIs reported were calculated around their respective effect sizes.

Behaviour. Active and alternative lever responses were analyzed using separate independent samples *t*-tests, with *feeding condition* (Sated, FDR) serving as the independent variable.

Western blot assays. Adjusted density values were analyzed separately for each brain region using two-way between subjects ANOVAs, with *feeding condition* (Sated, FDR) and *testing condition* (Naïve, Not-Tested, Tested) serving as the between subjects factors.

3.4 RESULTS

All heroin-trained rats acquired reliable heroin self-administration behaviour, as supported by the mean ± SEM number of infusions (37.63±3.29), active (121.47±17.08) and alternative (10.53±1.83) lever responses made on the last day of heroin self-administration training. There were no statistically significant differences in any of the reported parameters between the different experimental groups within each experiment.

Three rats were removed due to catheter leakage. Therefore, the final analyses were conducted on 60 rats in six experimental groups: Sated-Naïve (n = 11), Sated-Not-Tested (n = 6), Sated-Tested (n = 13), FDR-Naïve (n = 11), FDR-Not-Tested (n = 7), and FDR-Tested (n = 12). **Table 3.1** presents the average BW of the rats in each group on test day or day of euthanasia. The average BWs of rats in the Sated group was statistically significantly greater than that of rats in the FDR group, within the Naïve ($t_{(20)}$ = 13.59, p < .001, d = 5.80, CI = [3.81, 7.75]), Not-Tested ($t_{(11)}$ = 10.45, p < .001, d = 5.80, CI = [3.16, 8.42]), and Tested ($t_{(23)}$ = 11.25, p < .001, d = 4.50, CI = [2.98, 6.00]) conditions.

	Sated	**FDR**
Naïve	419.55±5.59	326.00±4.01
Not-tested	444.33±8.81	329.86±6.79
Tested	438.38±6.80	321.33±7.94

Table 3.1. Mean ± SEM body weights for the sated and food-restricted rats across all experimental conditions.

Behaviour. Planned comparisons on test day (W14) revealed a statistically significant augmentation in active lever responses in the FDR group compared to the Sated group ($t_{(23)}$ = 3.03, p = .006, d = 1.21, CI = [0.34, 2.06]). No statistically significant differences were observed for alternative lever responses ($t_{(23)}$ = 1.75, p = .094, d = 0.70, CI = [-0.12, 1.50]; **Figure 3.1B**).

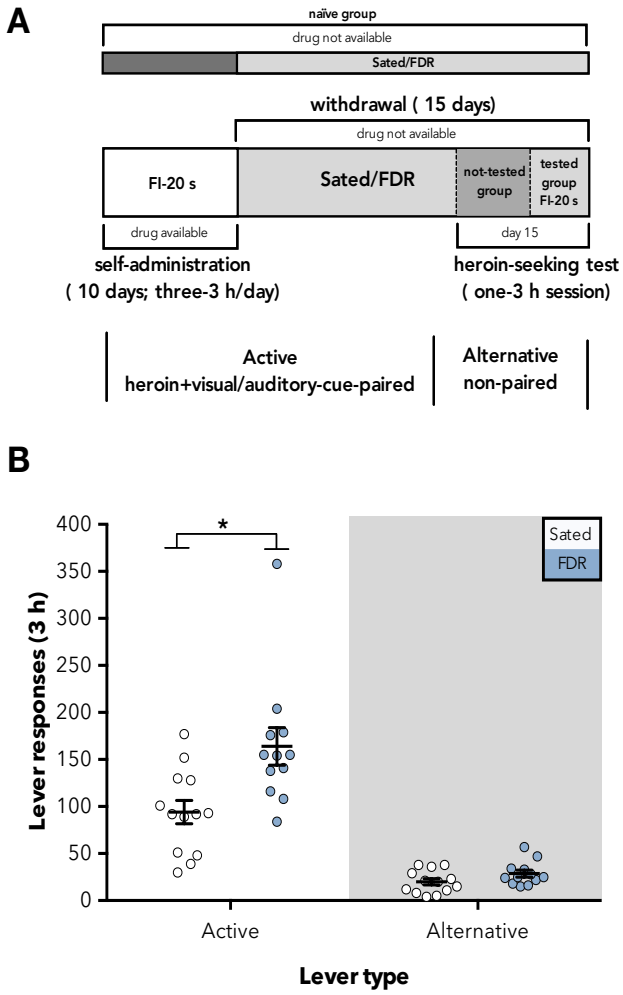

Figure 3.1. A food-restriction-induced augmentation of heroin seeking in rats under withdrawal. (**A**) Following heroin self-administration training rats underwent 15 days of withdrawal under food-restricted or sated conditions. Rats in the ***tested group*** underwent a 1-h heroin-seeking test under extinction conditions, and sacrificed immediately after. Rats in the ***non-tested group*** were sacrificed immediately prior to testing. A third group of rats that did not undergo surgery or self-administration training (***naïve group***) was handled and weighed daily for 11 days, exposed to 14 days of unrestricted or restricted access to chow, and then euthanized on day 14. (**B**) The number of active and alternative lever responses (±SEM) during the heroin-seeking test (*tested group;* FDR n = 12; Sated n = 13).*****p = .002.

Western blot assays. Western assays were conducted on a subset of animals from each group. For a complete list of the group sizes used in each assay, refer to **Table 3.2**.

Nucleus accumbens shell. Differences in GluA1 levels were observed across *testing conditions* ($F_{(2, 31)}$ = 3.47, p = .044, η^2 = 0.17, CI = [0.003, 0.32]). Specifically, a statistically significant decrease in GluA1 levels was observed in the rats who underwent heroin self-administration training, but did not undergo a heroin-seeking test (Not-Tested group) compared to Naïve rats ($t_{(31)}$ = 2.62, p = .040, d = 1.09, CI = [0.21, 1.96]). Although not statistically significant, a decrease in GluA1 levels in the Not-Tested group compared to rats who were exposed to a heroin-seeking test (Tested group) was supported by a large effect size ($t_{(31)}$ = 1.69, p = .273, d = 0.74,

CI = [-0.16, 1.63]). No statistically significant differences were observed between the Naïve and Tested groups ($t_{(31)}$ = 0.91, p = .752, d = 0.36, *CI* = [-0.43, 1.15]). Furthermore, food restriction did not have a statistically significant effect on GluA1 levels (*feeding condition;* $F_{(1, 31)}$ = 0.17, p = .680, η^2 = 0.004, *CI* = [0.00, 0.10]), and no statistically significant effects were observed for the *feeding condition X testing condition* interaction ($F_{(2, 31)}$ = 1.64, p = .211, η^2 = 0.08, *CI* = [0.00, 0.23]) was observed (**Figure 3.2A**).

A similar pattern was observed for p-Ser845-GluA1 levels, where statistically significant differences in *testing condition* ($F_{(2, 31)}$ = 4.33, p = .022, η^2 = 0.21, *CI* = [0.02, 0.36]) were found. In particular, a statistically significant decrease in p-Ser845-GluA1 levels was found for the Not-Tested rats, compared to the Naïve ($t_{(31)}$ = 2.63, p = .039, d = 1.06, *CI* = [0.21, 1.90]) and Tested ($t_{(31)}$ = 2.62, p = .040, d = 1.45, *CI* = [0.20, 2.06]) groups. No statistically significant differences were observed between the Naïve and Tested groups ($t_{(31)}$ = 0.21, p = .995, d = 0.08, *CI* = [-0.69, 0.85]). Furthermore, food restriction did not have a statistically significant effect on p-Ser845-GluA1 levels (*feeding condition;* $F_{(1, 31)}$ = 0.69, p = .414, η^2 = 0.02, *CI* = [0.00, 0.14]), and no statistically significant effects were observed for the *feeding condition X testing condition* interaction ($F_{(2, 31)}$ = 0.43, p = .660, η^2 = 0.02, *CI* = [0.00, 0.12]; **Figure 3.2B**).

Nucleus accumbens core. No statistically significant main effects were observed for the *feeding condition* (GluA1: $F_{(1, 13)}$ = 0.27, p = .610, η^2 = 0.02, *CI* = [0.00, 0.19]; p-Ser845-GluA1: $F_{(1, 39)}$ = 2.30, p = .138, η^2 = 0.05, *CI* = [0.00, 0.18]) and *testing condition* (GluA1: $F_{(2, 13)}$ = 0.62, p = .551, η^2 = 0.08, *CI* = [0.00, 0.24]; p-Ser845-GluA1: $F_{(2, 39)}$ = 0.64, p = .534, η^2 = 0.03, *CI* = [0.00, 0.12]). Furthermore, no statistically significant *feeding condition* X *testing condition* interactions (GluA1: $F_{(2, 13)}$ = 0.43, p = .660, η^2 = 0.06, *CI* = [0.00, 0.20]; p-Ser845-GluA1: $F_{(2, 39)}$ = 0.26, p = .773, η^2 = 0.01, *CI* = [0.00, 0.072]) were observed (**Figure 3.2C-D**).

Prelimbic cortex. No statistically significant main effects were observed for the *feeding*

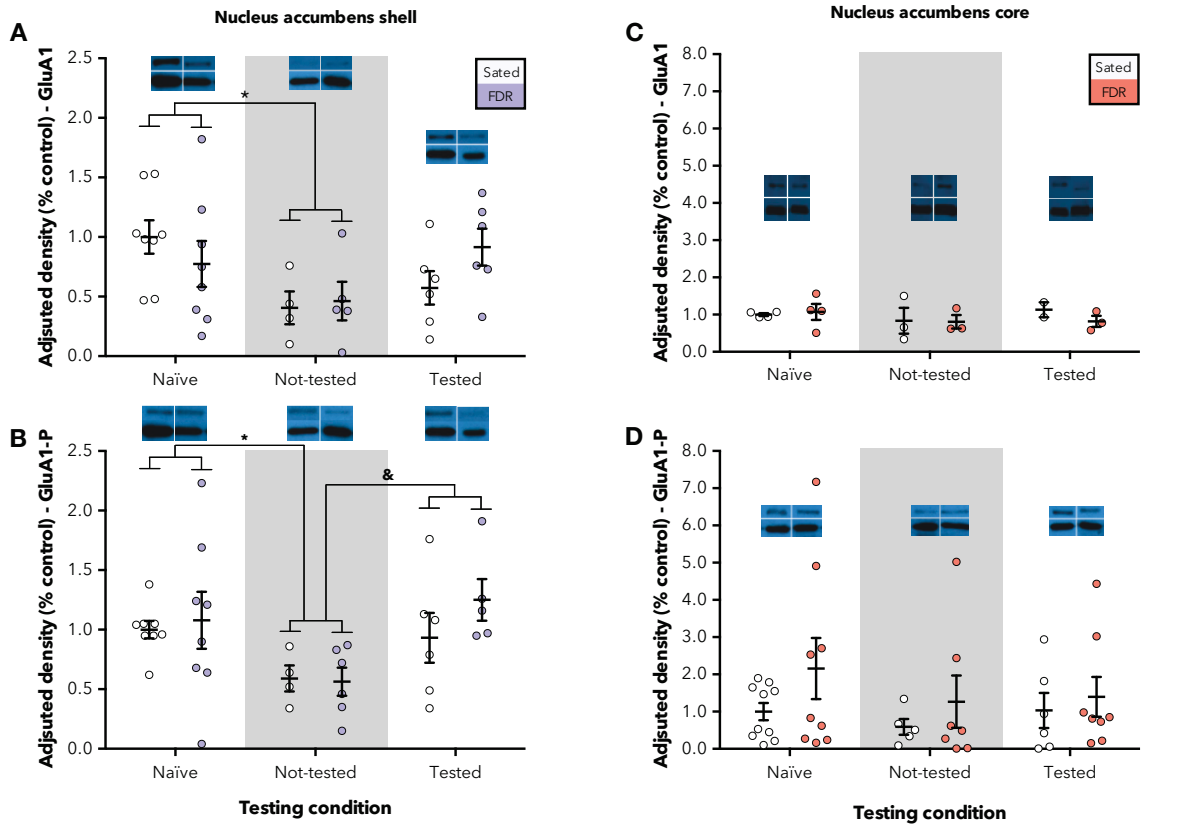

Figure 3.2. Re-exposure to a heroin context following self-administration, and a prolonged period of withdrawal increase GluA1 protein densities in the nucleus accumbens shell. All adjusted protein densities, taken as a percentage of control (Sated-naïve group) are presented as mean (±SEM). Upper bands represent the targeted protein for each group, while the lower bands represent the β-actin control. *Naïve* rats were subjected to a 14 day period in which half the rats were food-restricted. *Not-tested* rats underwent heroin self-administration, a 14 day period of withdrawal in which half of the rats were food-restricted and never exposed to a heroin-seeking test. Tested rats were treated similarly to those in the not-tested group except that they underwent one 1 h heroin-seeking test under extinction conditions. (**A-B**) Adjusted protein densities of GluA1 (Sated naïve, $n = 8$; Sated not-tested, $n = 4$; Sated tested, $n = 6$; FDR naïve, $n = 8$; FDR not-tested, $n = 5$; FDR tested, $n = 6$), and phosphorylated GluA1 (Sated naïve, $n = 8$; Sated not-tested, $n = 4$; Sated tested, $n = 6$; FDR naïve, $n = 8$; FDR not-tested, $n = 6$; FDR tested, $n = 5$) levels in the nucleus accumbens shell of food-restricted and sated rats, across testing conditions. (**C-D**) Adjusted protein densities of GluA1 (Sated naïve, $n = 4$; Sated not-tested, $n = 3$; Sated tested, $n = 2$; FDR naïve, $n = 4$; FDR not-tested, $n = 3$; FDR tested, $n = 3$), and phosphorylated GluA1 (Sated naïve, $n = 10$; Sated not-tested, $n = 5$; Sated tested, $n = 6$; FDR naïve, $n = 9$; FDR not-tested, $n = 7$; FDR tested, $n = 8$) levels in the nucleus accumbens core of food-restricted and sated rats, across testing conditions.*$p < .05$.

condition (GluA1: $F_{(1, 17)} = 1.91$, $p = .185$, $\eta^2 = 0.003$, $CI = [0.00, 0.29]$; p-Ser845-GluA1: $F_{(1, 12)}$

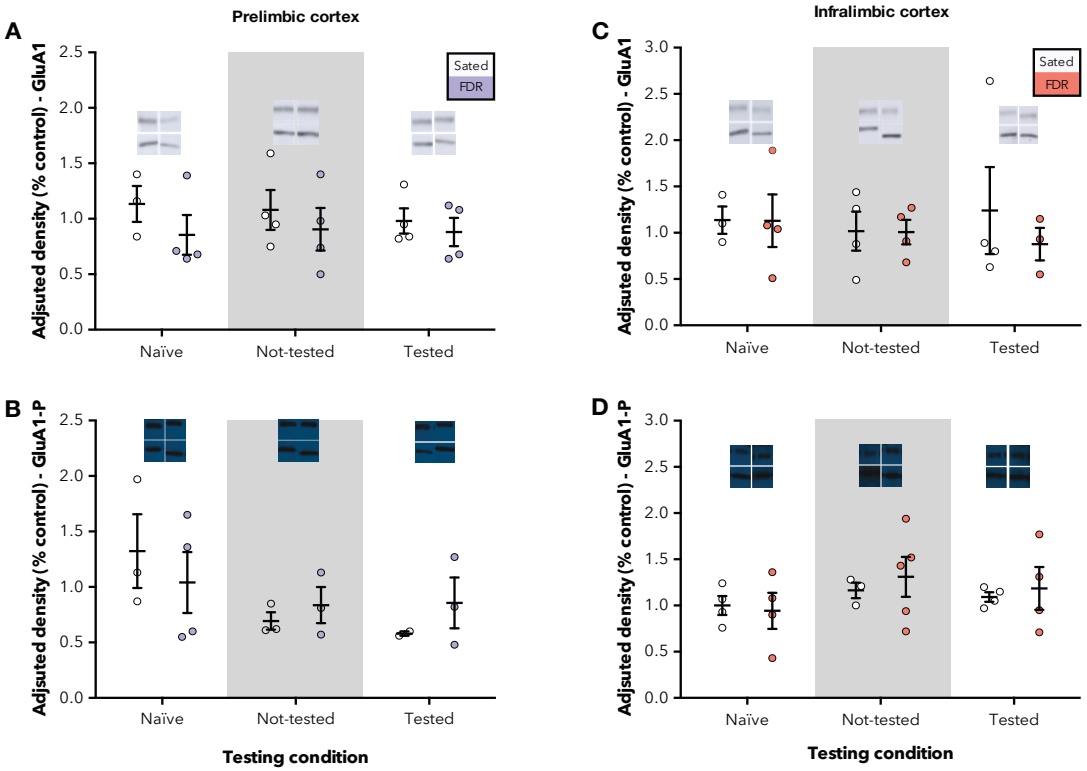

Figure 3.3. No changes in GluA1 levels observed between sated and food-restricted rats across multiple testing conditions. All adjusted protein densities, taken as a percentage of control (Sated-Naïve group) are presented as mean (±SEM) protein levels. Upper bands represent the targeted protein for each group, while the lower bands represent the β-actin control. *Naïve* rats were subjected to a 14 day period in which half of the rats were food-restricted. *Not-tested* rats underwent heroin self-administration, a 14 day period of withdrawal in which half of the rats were food-restricted and never exposed to a heroin-seeking test. Tested rats were treated similarly to those in the not-tested group except that they underwent one 1 h heroin-seeking test under extinction conditions. (**A-B**) Adjusted protein densities of GluA1 (Sated naïve, *n* = 3; Sated not-tested, *n* = 4; Sated tested, *n* = 4; FDR naïve, *n* = 4; FDR not-tested, *n* = 4; FDR tested, *n* = 4), and phosphorylated GluA1 (Sated naïve, *n* =3; Sated not-tested, *n* = 3; Sated tested, *n* = 2; FDR naïve, *n* = 4; FDR not-tested, *n* = 3; FDR tested, *n* = 3) levels in the prelimbic cortex of food-restricted and sated rats, across testing conditions. (**C-D**) Adjusted protein densities of GluA1 (Sated naïve, *n* = 3; Sated not-tested, *n* = 4; Sated tested, *n* = 4; FDR naïve, *n* = 4; FDR not-tested, *n* = 4; FDR tested, *n* = 3), and phosphorylated GluA1 (Sated naïve, *n* = 4; Sated not-tested, *n* = 3; Sated tested, *n* = 4; FDR naïve, *n* = 4; FDR not-tested, *n* = 5; FDR tested, *n* = 4) levels in the infralimbic cortex of food-restricted and sated rats, across testing conditions.*$p < .05$.

. 903, $\eta^2 = 0.27$, *CI* = [0.00, 0.06]; p-Ser845-GluA1: $F_{(2, 12)} = 2.33$, $p = .139$, $\eta^2 = 0.01$, *CI* = [0.00, 0.44]). Furthermore, no statistically significant *feeding condition* X *testing condition* interactions (GluA1: $F_{(2, 17)} = 0.15$, $p = .886$, $\eta^2 = 0.09$, *CI* = [0.00, 0.09]; p-Ser845-GluA1: $F_{(2, 12)} = 0.75$, $p = .494$, $\eta^2 = 0.02$, *CI* = [0.00, 0.27]) were observed (**Figure 3.3A-B**).

Infralimbic cortex. No statistically significant main effects were observed for the *feeding condition* (GluA1: $F_{(1, 15)} = 0.30$, $p = .594$, $\eta^2 = 0.02$, $CI = [0.00, 0.17]$; p-Ser845-GluA1: $F_{(1, 18)} = 0.17$, $p = .682$, $\eta^2 = 0.008$, $CI = [0.00, 0.14]$) and *testing condition* (GluA1: $F_{(2, 15)} = 0.09$, $p = .912$, $\eta^2 = 0.01$, $CI = [0.00, 0.05]$; p-Ser845-GluA1: $F_{(2, 18)} = 1.13$, $p = .345$, $\eta^2 = 0.11$, $CI = [0.00, 0.26]$). Furthermore, no statistically significant *feeding condition* X *testing condition* interactions (GluA1: $F_{(2, 15)} = 0.25$, $p = .780$, $\eta^2 = 0.03$, $CI = [0.00, 0.13]$; p-Ser845-GluA1: $F_{(2, 18)} = 0.18$, $p = .840$, $\eta^2 = 0.02$, $CI = [0.00, 0.15]$) were observed (**Figure 3.3C-D**).

3.5 DISCUSSION

Alterations in synaptic plasticity are believed to underlie the development of drug addiction and the vulnerability to relapse. In addition to what has been previously reported by our laboratory (Sedki, Abbas, Angelis, Martin, et al., 2013a), we have also demonstrated augmented heroin seeking in chronically food-restricted rats under withdrawal. Re-exposure to cues previously associated with heroin self-administration and the opportunity to seek heroin following withdrawal (tested group) elicited an increase in p-Ser845-GluA1 expression within the NAc shell, compared to similarly trained, but not tested rats. A reduction in GluA1 and p-Ser845-GluA1 subunit expression was observed in the not-tested group compared to drug-naïve control rats, while no changes in NAc shell expression were observed between the naïve and tested groups, nor across feeding conditions. Finally, no differences were found in total and p-Ser845-GluA1 subunit expression between testing or feeding conditions in the NAc core, IL, or PL subregions.

3.5.1 Nucleus accumbens GluA1 receptor subunit expression in heroin-trained rats under withdrawal.

It has been previously reported that 14 days of non-contingent morphine injections decreased the expression of the GluA1 receptor subunit on NAc shell MSNs (Glass et al., 2008). Despite differences in the drug administration protocol (i.e., experimenter administered vs. self-administration) and withdrawal time (i.e., 1 h vs. 14 days), the findings correspond with the reduced NAc shell GluA1 and p-ser845-GluA1 expression in the not-tested (vs. naïve) rats, suggesting that the observed decrease may be specific to opiate exposure. However, this conclusion contrasts with findings indicating greater level of NAc shell GluA1 expression 45 days, compared to 1 day, following the cessation of cocaine self-administration (Conrad et al., 2008). This lack of consensus may reflect differences in psychostimulant and opiate exposure, or the methodological diversity between studies.

Opposing changes in AMPAR expression have been suggested in response to stimulant and opiate exposure. Withdrawal from passive, chronically-administered cocaine has been associated with LTP-like changes within the NAc. Such changes include an increase in cell surface expression of the GluA1 subunit (Boudreau & Wolf, 2005), increased spine head diameters (Shen et al., 2009) and augmented AMPAR-mediated currents (Kourrich et al., 2007), suggesting enhanced synaptic plasticity. In contrast, the reductions described above in accordance with reported reductions in dendritic spine density in the NAc following chronic morphine self-administration (Robinson, Gorny, Savage, & Kolb, 2002), and the attenuated AMPAR-mediated currents observed in heroin-treated rats indicate a depression of synaptic plasticity (Shen & Kalivas, 2013). However, we should note that the simple contrast in GluA1 subunit expression believed to drive the diverse changes in receptor currents may appear to be more complex than previously theorized. For example, the expression of glutamate receptors on NAc shell MSNs is thought to depend on the MSN subpopulations. Following repeated morphine injections, synaptic GluA2-lacking AMPAR expression increased in MSNs co-expressing DA-D1-Rs but decreased in those co-expressing DA-D2-Rs. Given that increases and decreases in AMPAR expression in specific MSNs are observed simultaneously, then measuring the overall changes in receptor expression within the NAc shell may improperly illustrate the nature of the change in AMPARs (Hearing et al., 2016). Moreover, it is important

to consider the procedural disparity between studies. For instance, Hearing and colleagues (2016) inferred the changes in synaptic presence of GluA2-lacking AMPAR through slice electrophysiology, while we measured overall protein expression. Therefore, we cannot comment on the specific location of the proteins or on their trafficking.

Following the heroin-seeking test, we report greater p-ser845-GluA1 subunit expression in the NAc shell, compared to the not-tested group. We believe this increase reflects rapid phosphorylation of the receptor subunit in response to heroin cues, and not an increase in total receptor expression as those levels remained unchanged. This contradicts the findings of Conrad et al. (2008), who report no changes in cell surface or intracellular expression of the GluA1 receptor subunit in rats exposed to a cocaine-seeking test following 45 days of withdrawal, compared to cocaine-trained, non-tested rats sacrificed on the same day. The inconsistencies outlined here may be due to differences in drug type, the length of the withdrawal period, or the methodological distinctions in the protein quantification procedures employed. For example, we did not discover any changes in total GluA1 subunit expression and our changes related specifically to the phosphorylation of the receptor subunit. While phosphorylation is known to facilitate receptor trafficking (Oh, Derkach, Guire, & Soderling, 2006), increases in the phosphorylation of a receptor do not guarantee that surface trafficking will occur. No statistically significant differences were observed between rats exposed to a heroin-seeking and drug-naïve control rats. This may reflect underlying differences in the distribution of the glutamatergic receptors within each group (i.e. synaptic, extra-synaptic, cytoplasmic). Future studies are required to elucidate if such differences in receptor distribution are present.

No differences in GluA1 and p-Ser845-GluA1 subunit expression were characterized within the NAc core, a finding which is in disagreement with the increased GluA1 protein expression observed three weeks following a single injection of morphine (Jacobs, Wardeh, Smit, & Schoffelmeer, 2005). However, we note that our rats did not undergo passive or acute opiate administration, and instead underwent 10 days of self-administration training. Consistent with,

and more relevant to present studies, is recent evidence that expression of the GluA1 subunit in the NAc core is unchanged in heroin-trained, compared to saline-trained rats after a period of extinction (Shen et al., 2011). Specifically, this effect reported by Shen (Shen et al., 2011) was accompanied by a diminished AMPAR/NMDAR ratio, and was driven by an increase in the NMDA NR2B receptor subunit surface expression and not AMPAR related changes. However, we advise caution in the comparison of our data with the Shen et. al (2011) study due to the difference in passive withdrawal versus the extinction procedure used by each laboratory, respectively.

3.5.2 Medial prefrontal cortex GluA1 receptor subunit expression in heroin-trained rats under withdrawal.

No differences in total and p-Ser845-GluA1 subunit expression were characterized within the IL or PL subregions. These effects are consistent with the overall findings that mPFC GluA1 subunit expression may not be altered following exposure to psychostimulants or opiates. Indeed, we have previously observed no changes in dorsomedial prefrontal cortex (dmPFC) GluA1 subunit expression following a food-deprivation-induced reinstatement of heroin seeking (Tobin & Shalev, 2012). In addition, no changes in the GluA1 and decreases in the GluA2 and GluA3 subunits were reported following a cue-induced heroin-seeking test in rats under withdrawal compared to non-tested rats (Van den Oever et al., 2008). Interestingly, while no changes in the GluA1 subunit were observed, a reduced AMPA/NMDA ratio driven by AMPAR current changes was reported in rats exposed to a cue-induced heroin reinstatement. Importantly, the authors suggest that this reduction was the outcome of GluA2 endocytosis within the ventromedial prefrontal cortex (vmPFC), as blocking this endocytosis has been shown to attenuate heroin reinstatement (Van den Oever et al., 2008).

3.5.3 Involvement of GluA1 receptor subunit expression in the food-restriction-induced augmentation of heroin seeking in rats under withdrawal.

The lack of any statistically significant feeding-related changes in GluA1 expression within the NAc shell, the NAc core, the IL and the PL, is consistent with findings from our laboratory. Specifically, we have reported that an acute 21 h food deprivation failed to alter the expression of the GluA1 subunit in the NAc or dmPFC in drug-naïve rats or rats exposed to a food-deprivation-induced heroin reinstatement (Tobin & Shalev, 2012). The latter findings are relevant to the lack of changes observed here among the naïve and tested groups, with an important distinction. Particularly, rats in the current study did not undergo extinction, therefore any comparison must be interpreted with caution as clear differences in the neuronal basis of extinction and withdrawal exist (Fuchs et al., 2006). In addition, recent data from our laboratory using *in vitro* whole cell patch-clamp recordings demonstrated that drug-naïve rats subjected to 14 days of chronic food restriction show a marked reduction in NMDAR, but not AMPAR currents, resulting in increased AMPAR/NMDAR ratios relative to drug-naïve, sated controls (Glovaci, D'Cunha, Chapman, & Shalev, 2018). These findings suggest that food restriction may alter NMDAR, but not AMPAR, functioning. Though it is important to consider that the findings gathered through electrophysiological techniques reflect functional evidence, the data reported here were collected through biochemical assays, and changes in AMPAR currents do not always follow cell surface trafficking or total protein expression changes.

In contrast with the lack of changes observed here, Jung and colleagues (2016) have reported elevated NAc shell and NAc core p-Ser845-GluA1-R protein expression in chronically food-restricted, compared to sated rats exposed to a morphine-paired environment. We believe that the resulting disparity underlies the discrepancy between operant (drug seeking) and Pavlovian (CPP) measures of drug-cue reward. In addition, the null effects described above contradict multiple demonstrations that, compared to sated controls, drug-naïve food-restricted rats exhibit increased p-ser845-GluA1 and GluA1, but not GluA2 or GluA3 expression in the NAc (Carr, 2016; Ouyang et al., 2017; Peng et al., 2015). Furthermore, our restriction procedure imposed a gradual 10% decrease in BW over 14 days, while the procedure imposed by Carr's (2016; 2015) group demanded a 20% decrease in BW over one week, followed by maintenance

for an additional week. Thus, we speculate that variability in our findings result from methodological differences, though this has yet to be directly examined.

Increases in p-ser845-GluA1 and GluA1 expression are also accompanied by a decrease in calcineurin levels, which are thought to be necessary for GluA1 endocytosis and p-Ser-845-GluA1 dephosphorylisation (Carr, 2016). Importantly, this decrease can facilitate the trafficking of GluA1 to the cell surface. Therefore, it is conceivable that one method by which food restriction may potentiate the rewarding effects of appetitive stimuli is by enhancing the synaptic incorporation of calcium permeable GluA1 AMPARs into the cell surface. Thus, the effects of glutamate are most likely governed by the availability of iGluR subunits on the cell surface rather than intracellular deposits, and the trafficking of such receptors is crucial for synaptic plasticity (Boudreau & Wolf, 2005). The findings reported by Carrs group (Carr, 2016; Ouyang et al., 2017; Peng et al., 2015) used subcellular fractionation and cross-linking techniques to measure expression within the post synaptic densities on the cell surface of the membranes, whereas the procedures employed here allowed only for total protein expression. The lack of fraction specificity may therefore account for the differences in findings.

3.5.4 Conclusions

The aforementioned experiments were designed to evaluate GluA1 and p-Ser845-GluA1 subunit expression changes in chronically food-restricted, heroin-seeking rats. While we did indicate that prolonged withdrawal from heroin training specifically reduces GluA1 and Ser-845-GluA1 NAc shell subunit expression, to our surprise, we failed to identify any statistically significant differences across feeding conditions. Due to strong evidence that food restriction may modify the synaptic incorporation of AMPARs, and the behavioural response to drug-associated cues (Carr, 2016; Ouyang et al., 2017), we are hesitant to suggest that chronic food restriction does not produce meaningful changes in GluA1 subunit expression. Instead, we believe our null findings reflect a limitation of the molecular procedures employed. Therefore, we recommend that future studies apply subcellular fractionation or BS^3 protein cross-linking

techniques, which have been developed to dissociate membrane-bound and cytoplasmic receptors (Boudreau & Wolf, 2005).

Chapter 4: What about the cue? Modulation of cue value in the augmentation of heroin seeking in chronically food-restricted rats under withdrawal

4.1 ABSTRACT

Rationale. Disruptions in energy balance can affect motivated behaviours. For example, caloric restriction can increase drug taking and seeking. Recently, we reported augmented heroin seeking in food-restricted rats under withdrawal. The underlying motivational mechanisms, however, remain unclear. It is possible that exposure to caloric restriction enhances the incentive value attributed to drug-associated cues, and in turn, augment drug seeking. However, it is important to distinguish the incentive value of a light cue independent of the value acquired through its association with a drug.

Objectives. We investigated the effects of food restriction on the acquisition of a new operant response reinforced solely by heroin- or non-heroin-associated cues. We have also compared heroin- with non-heroin-associated light seeking in a discrete choice paradigm.

Methods. Male Long-Evans rats were trained (10 days) to lever-press for heroin. Next, rats were moved to the animal colony and maintained on free access to food (Sated) or subjected to 14 days of mild food restriction (FDR). On day 14, rats were tested in the self-administration chambers. Experiment 1: During self-administration training, heroin infusions were associated with light/tone cues. On test day, the incentive value of the cues was assessed by training rats on a novel nose poke response reinforced solely by the heroin cues. Experiment 2: Responses on one lever led to a heroin infusion, and the activation of a light/tone cue. Responses on a second lever activated a different light/tone or just a tone cue. On test day, rats were treated as described in Experiment 1. Experiment 3: Rats were trained on a discrete choice trial, with one lever associated with heroin infusions+light cue, and another lever associated only with a different light cue. On test day rats were exposed to choice trials reinforced by cues alone.

Results. Experiment 1: Rats in the FDR group acquired the novel behaviour at a greater level compared to the Sated group. Experiment 2: A food-restriction–induced increase in nose poke response rate was observed for both heroin-paired and the alternative light cue. Experiment 3: Responses for a heroin-associated light cue was greater than responses on for the light cue

alone in both Sated and FDR groups. Response rate for the light alone however was greater in the FDR versus Sated group.

Conclusions. Our findings suggest that food restriction increases the conditioned motivational properties of environmental stimuli, including, but not exclusive to, heroin-paired cues.

Keywords Food restriction · Heroin seeking · Motivation · Incentive value · Acquisition of novel behaviour · Light seeking

4.2 INTRODUCTION

Neutral stimuli that have been repeatedly paired with the pharmacological effects of abused drugs often come to acquire conditioned reinforcing properties. These conditioned reinforcers are thought to gain incentive motivational properties that perpetuate drug taking, drug craving, and the propensity to return to drug seeking in animal models of relapse (Robinson & Berridge, 1993). Indeed, during self-administration training, the coupling of conditioned reinforcers with drug delivery can increase a rat's willingness to respond for the drug (Caggiula et al., 2001; Deroche-Gamonet et al., 2002). When the drug is no longer available, encounters with drug-associated cues can both elicit and strengthen the vigor with which the previously self-administered drugs were sought (de Wit & Stewart, 1981b; Di Ciano & Everitt, 2004; Shaham et al., 2003). Furthermore, reintroduction to an environment previously paired with heroin or cocaine, and exposure to discrete drug-associated cues have all been shown to successfully reinstate extinguished drug seeking (Crombag et al., 2008a; Lee et al., 2006; Shalev et al., 2002; Stewart, 2000). Moreover, responses reinforced by drug-conditioned stimuli increase over time and remain elevated long after physical withdrawal from the drug has abated, suggesting a time-dependent incubation of their motivational attributes (Grimm et al., 2001; Pickens et al., 2011; Venniro et al., 2016).

Interestingly, recent evidence has indicated that the incentive motivation attributed to conditioned reinforcers may be further modulated by one's current need state. For example, in a hunger state, the value of food-predictive stimuli may increase (Aitken, Greenfield, & Wassum, 2016). Although this state-dependent behaviour may be adaptive for survival under particular circumstances, it might lead to compulsive food or drug seeking (Corbit, Janak, & Balleine, 2007; Hogarth, Balleine, Corbit, & Killcross, 2013; Milton & Everitt, 2012; Ostlund & Balleine, 2008).

The effects of hunger on the motivational salience of non-food related reward have been demonstrated in chronically food-restricted rats, where reduced thresholds for electrical BSR

reward are observed (Fulton et al., 2000). This is consistent with evidence supporting a modulatory role for food restriction on drug-related behaviours. Specifically, it has been reported that caloric restrictions influence drug rewarding properties, drug self-administration and the conditioned reinforcing properties of abused drugs (Carr, 2007; Stuber et al., 2002). Recent work in our laboratory has also revealed an augmentation of heroin seeking in chronically food-restricted rats under prolonged withdrawal (D'Cunha et al., 2013). Currently, the psychological processes that underlie the food-restriction-induced augmentation of heroin seeking are unknown.

One possibility is that the incentive motivational properties of heroin cues increase under food restriction conditions. However, another possibility states that food restriction may enhance the inherent motivational value of all cues, irrespective of their drug-conditioned reinforcing properties. Studies investigating the role of food restriction on the reinforcing value of visual stimuli have revealed greater lever pressing for a visual stimulus in hungry rats (Davis, 1958; Hurwitz & De, 1958; Segal, 1959; Stewart, 1960), though other studies have reported contradictory findings (Hurwitz & De, 1958; Smith & Donahoe, 1966). More recently, Keller and colleagues (2014) reported that lever pressing reinforced by visual stimuli (i.e., white cue light) was moderately potentiated by food restriction, though the contribution of visual stimuli to drug seeking (rather than drug taking) under both sated and food-restricted conditions has yet to be elucidated. Generally, limited information exists on the effects of visual stimuli in opioid drug-seeking procedures. Thus, it is unclear whether the effects of chronic food restriction on drug seeking result from the attribution of incentive motivational properties to the drug-paired cues, or from a generalized increase in the reinforcing properties of the visual cues used in our instrumental procedure.

The present study was designed to address two questions. First, we asked whether the incentive motivational properties of heroin cues are increased in food-restricted rats. Second, we examined whether the effects of food restriction are exclusive to heroin-paired cues. The incentive motivational properties of heroin-associated cues in food-restricted rats were

investigated using two procedures. In one procedure, rats were tested under a PR schedule of reinforcement, which is commonly used to study reward motivation in rodents (Hodos, 1961; Richardson & Roberts, 1996). In addition, conditioned stimuli have been shown to support the novel learning of instrumental actions (Di Ciano, 2008; Di Ciano & Everitt, 2004; Samaha et al., 2011). Therefore, the acquisition of a novel operant response reinforced solely by the heroin-associated cues was used to investigate the conditioned rewarding properties of such cues. To confirm that the augmentation of heroin seeking in food-restricted rats resulted from the heroin-cue pairing, and not the inherent motivational properties of visual cues, we conducted three experiments. First, we attempted to replicate the food-restriction-induced augmentation of responses for visual cues with the compound light/tone cues that are routinely used during drug self-administration training (Keller et al., 2014). Next, we investigated whether the inclusion of a neutral, non-heroin-paired cue alternative would alter behaviour during the acquisition of a novel operant response reinforced by heroin-paired cues. Lastly, we presented food-restricted rats with discrete choice trials, in which both heroin-paired and alternative neutral cues were available. We hypothesized that food restriction would increase the incentive value attributed to heroin-paired cues versus neutral cues, when compared to sated controls.

4.3 METHODS

4.3.1 Subjects

A total of 152 male, Long Evans rats (Charles River, St Constant, Quebec, Canada or New Jersey, USA; 275-300) were used. Before surgery, rats were pair-housed and handled for one week in the ACF under 12 h reverse light/dark conditions (lights OFF at 0930 h). Following IV catheterization, rats were single-housed in plastic shoebox cages and allowed two or four days of recovery. Next, rats were transferred to operant conditioning chambers for heroin self-administration. Following self-administration training, rats were returned to the ACF and single-housed in shoebox cages for the drug-washout and withdrawal periods. Rats were then returned to the operant conditioning chambers for testing. Except for the withdrawal and testing phases, all rats were allowed 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.

4.3.2 Surgical Procedures

With the exception of rats in Experiment 3, all rats were implanted with IV silastic catheters (Dow Corning, Midland, MI, USA) under xylazine+ketamine (13.0+90.0 mg/kg; ip) anesthesia, as described previously (Sedki, D'Cunha, & Shalev, 2013b). Following surgery, rats were administered saline (0.9%) for hydration, penicillin (450 000 IU/rat; s.c.) to prevent infection and an analgesic (buprenorphine, 10.0 µg/kg; s.c.; Schering-Plough Ltd., Welwy Garden City Hertfordshire, UK; ketoprofen, 2.0 mg/kg; s.c.; CDMV, QC, CA). Ketoprofen was administered for 2 additional days following surgery. Catheters were flushed daily throughout the self-administration phase, with heparin+gentamicin (7.5 UI + 12.0 mg/rat) to prevent blockage and infection.

4.3.3 Apparatus

Experiment 1, 5. During self-administration and testing, rats were individually housed in operant conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 X 29.0 X 25.5 cm) enclosed in sound attenuating wooden cubicles, each equipped with a fan (noise rating of 45 dBA; Newark, Canada). The front and back walls of the chambers were built using polycarbonate plexiglass, while the ceiling and side walls were composed of stainless steel. Each chamber was fitted with two retractable levers mounted 9 cm above the metal grid floor of the right sidewall. All rats were attached to an infusion pump (3.33 rpm), located above the chamber, via a liquid swivel (Lomir Biomedical Inc., Notre-Dame-de-L'île-Perrot, QC, Canada) and Tygon tubing (VWR Canada) shielded with a metal spring. ***Experiment 1:*** A red house light was installed at the top center of the left sidewall, while a white cue-light and a tone generator (Coulbourn Instruments; Sonalert, 2.9 KHz) were located directly above the active (heroin+visual/auditory-cue-paired) lever. ***Experiment 5:*** A red house light was located at the top center of the left sidewall, while one of two cue lights (flashing blue light, white light) was

located either directly above the active (heroin+visual-cue-paired) or the alternative (visual-cue-paired) lever.

Experiment 2, 3, 4A, 4B. During self-administration and testing, rats were individually housed in operant conditioning chambers (Med Associates Inc, St. Albans, Vermont, USA; 32.0 X 24.0 X 25.0 cm) enclosed in sound attenuating wooden compartments, each equipped with a fan. The ceiling, front and back walls of the chamber were constructed from polycarbonate plexiglass, while the left and right sidewalls were built using stainless steel. Each chamber was fitted with two levers or nose poke modules (heroin-seeking test, Experiments 2 & 4) mounted 5 cm above the metal grid floor of the right sidewall. All rats (except those in Experiment 3) were attached to an infusion pump (1.00 rpm), located above the chamber, via a liquid swivel and Tygon tubing shielded with a metal spring. House and cue light arrangements differed across experiments. ***Experiment 2:*** A red house light was installed at the top center of the left sidewall, and a cue-light and a tone generator were located directly above the active (heroin+visual/auditory-cue-paired) lever. ***Experiment 3:*** A red house light was installed at the top center of the left sidewall and a cue-light and a tone generator were located directly above the active (visual/auditory-cue-paired) lever. ***Experiment 4:*** Two visual (white light, red light) and three different auditory (2.9 KHz tone, white noise, clicker) cues were used, and counterbalanced across rats. Different visual-cue and sound generator combinations were installed directly above the active (heroin+visual/auditory-cue-paired), and alternative (*4A:* visual/auditory-cue-paired, *4B:* auditory-paired) levers.

4.3.4 Drug

Heroin HCl (a generous contribution from the National Institute for Drug Abuse, Research Triangle Park, NC, USA) was dissolved in sterile saline to yield a dose of 0.1 mg/kg/infusion adjusted for each rat according to BW.

4.3.5 Procedure

Experiment 1: The effects of chronic food restriction on lever pressing reinforced by heroin-associated cues under a progressive ratio schedule of reinforcement.

Self-Administration (10 days). Following recovery from surgery, rats were allowed a 24 h habituation period to the operant conditioning chambers. Next, rats were trained to self-administer heroin in daily, three, 3 h sessions separated by 3 h intervals for 10 days. The first session began within 30 min after the onset of the dark phase with the extension of the levers into the operant conditioning chamber, illumination of the red house light and activation of the compound visual/auditory cues for 30 s. Responses on the active (heroin+visual/auditory-cue-paired) lever resulted in activation of the drug pump (5 s; 0.13 ml/infusion) followed by a 20 s timeout period, during which the house light was turned off, the compound visual/auditory cues were activated, and active lever responses were recorded but not reinforced. Following each 3 h session, the active lever was retracted whereas the alternative lever was only retracted 1 h prior to the first session on the next day. Alternative (non-paired) lever responses were recorded but had no programmed consequences.

Withdrawal (15 days). Following self-administration training, rats were individually housed in the ACF and allowed unrestricted access to food and water for one drug-washout day. Next, rats were divided into two groups: Sated, or food-restricted (FDR) that were matched according to BW, number of infusions, and active lever responses across the last five days of training. Following washout, on the second day of the withdrawal phase, rats in the FDR group had their food removed and were fed ~15 g of chow at 1330 h. The amount of food was adjusted throughout the 14 days of food restriction to maintain the food-restricted rats' BW at ~90% of their W1 BW.

Test (1 day). On W15, rats were returned to the operant conditioning chambers and attached to the metal spring. The heroin-seeking test consisted of one, 3 h session during which active lever responses were reinforced only by the heroin-associated compound visual/auditory cues, under a PR + 5 or 20 s timeout schedule of reinforcement. Thus, the number of active lever responses required to activate the compound visual/auditory cues increased after each presentation according to an exponential progression (PR schedule = 1, 2, 3, 5, 7, 9, 12 ,15, 18, 23, 28, 33, 41, 49, 57, 70, 83, 96, 117, 138, 156, 200, 225, 275, 300…) derived from the formula:

PR requirement = $[5e^{(\text{reward number X } 0.17)}]$-5 (Richardson & Roberts, 1996), rounded to the nearest integer. Responses during the timeout period were recorded but did not contribute toward the PR requirement. See **Figure 4.1A** for a summary of the experimental procedure.

Experiment 2: The effects of chronic food restriction on acquisition of a novel nose poke response reinforced by heroin-associated cues.

Self-Administration (10 days). Rats were treated as described in Experiment 1, with the exception of the drug pump which was activated for 12 s to yield a 0.13 ml infusion, and the alternative lever was non-retractable.

Withdrawal (23 days). Rats were treated identically to those in Experiment 1, with the exception of the withdrawal phase which lasted 23 days.

Heroin-seeking test (3 days). To assess whether food restriction increases the conditioned reinforcing properties of heroin-paired cues, we measured the acquisition of a novel nose poke response, which was only reinforced by the heroin-associated cues. Rats were returned to the operant conditioning chambers for a 3 h heroin-seeking test (W15, W18, and W23), and tethered to the metal spring. Self-administration levers were replaced with active and alternative nose poke devices (ENV-114 BM, Med Associates Inc.) located approximately 5 cm above the floor. The arrangement of the nose poke devices was counterbalanced; in half of the chambers, the active nose poke devices and associated cues were installed on the side of the alternative lever. Nose pokes were determined by disruptions of an infrared beam located within the aperture. Active and alternative nose pokes resulted in the same consequences as the corresponding levers during self-administration, excluding heroin delivery. See **Figure 4.2A** for a summary of the experimental procedure.

Experiment 3: The effects of chronic food restriction on instrumental responses reinforced by a compound visual/auditory cue.

Operant conditioning (10 days). Rats were treated similarly to those in Experiment 2, with a few exceptions. Specifically, they did not undergo surgery, they were not tethered to a metal spring, and the activation of the pump did not result in drug delivery. Responses on the active (visual/auditory-cue-paired) lever deactivated the house light, activated the pump (resulting in characteristic sound and vibrations), and turned on the compound visual/auditory cue located above the lever. Alternative (non-paired) lever responses were recorded but had no programmed consequences.

"***Withdrawal" (15 days).*** Rats were treated as described in Experiment 1.

Visual/auditory cue-seeking test (1 day). On "withdrawal" day 15, rats were returned to the operant conditioning chambers for a 3 h test, where responses on the active and alternative levers resulted in the same consequences as those during the operant conditioning phase. See **Figure 4.3A** for a summary of the experimental procedure.

Experiment 4: The effects of chronic food restriction on training for a novel nose poke response reinforced by heroin-paired or neutral cues.

To test whether the food-restriction-induced augmentation of nose poke responding (Experiment 2) was selective for heroin-paired cues (compared to neutral cues), visual and/or auditory cues were paired with responses on an alternative lever.

Self-administration (10 days). Daily session time, habituation, and heroin-delivery protocols were similar to those described in Experiment 2. However, rats underwent training in two phases, where the type of visual (white light; round plastic cover, red light; elongated metal cover) and auditory (tone, white noise, clicker) cues were counterbalanced across levers and chambers. ***Phase 1 (days 1-5):*** Each session began with the extension of the active lever into the operant conditioning chamber, and the 30 s activation of the visual/auditory compound cue that was paired with the active lever. Responses on the active lever resulted in the activation

of the drug pump (12 s; 0.13 ml/infusion). This was followed by a 30 s timeout during which the visual/auditory cue was activated, and additional active lever responses were recorded but not reinforced (FI-30 s). Following each 3 h session, the active lever was retracted whereas the alternative lever remained extended. Alternative (non-paired) lever responses were recorded but had no programmed consequences. ***Phase 2 (days 6-10):*** While the consequences of active lever responses remained identical to those described in phase 1, the alternative lever was programmed with a FI-30 s schedule of reinforcement. In Experiment 4A, responses on the alternative lever activated a compound visual/auditory cue positioned directly above the lever. In Experiment 4B, responses on the alternative lever resulted in the activation of an auditory cue directly above the lever.

Withdrawal (18 days). Rats were treated as described in Experiment 2.

Acquisition of novel response test (2 days). Rats were tested as described in Experiment 2, except that responses in the alternative nose poke aperture activated a visual/auditory cue (Experiment 4A) or auditory cue alone (Experiment 4B). See **Figures 4.4A and 4.5A** for summaries of the experimental procedures.

Experiment 5: The effects of chronic food restriction on the choice between heroin-paired cues and neutral cues.

Self-administration (19-20 days). Daily session time, habituation, and heroin-delivery protocols were identical to those described in Experiment 1. Our training procedure included three phases, designed to test choice preference for a heroin-paired or non-heroin-paired visual cue. ***Phase 1 (days 1-6):*** Presentations of the active (heroin+visual cue; days 1, 3, 5) or alternative (visual-cue-paired) lever (days 2, 4, 6) alternated daily. Each session began with the illumination of a red house light, the extension of the appropriate lever, and the activation of a visual cue (flashing blue light, constant white light; counterbalanced) located above each lever. Responses on either lever were reinforced with a FI-20 s schedule of reinforcement. Responses

on the active lever initiated heroin delivery and the illumination of the visual cue above the lever, while responses on the alternative (visual-cue-paired) lever activated a different visual-cue. ***Phase 2 (days 7-14):*** Presentations of the active (days 7, 9, 11, 13) or alternative (days 8, 10, 12, 14) levers alternated daily. Lever responses were now reinforced with a fixed ratio 1 + 5 min inter trial interval (FR1+5 min ITI) schedule of reinforcement. Active lever responses initiated heroin delivery, the illumination of the visual-cue for 20 s, the deactivation of the house light, and the retraction of the lever for 5 min. Responses on the alternative lever resulted in a 20 s illumination of the cue light, the deactivation of the house light, and the retraction of the lever for 5 minutes. ***Phase 3 (days 15-20):*** Rats were allowed daily access to both levers, under a FR1+5 min ITI schedule of reinforcement. Responses on the active lever resulted in heroin delivery, the illumination of its associated visual-cue for 20 s, the deactivation of the house light, and the retraction of both levers for 5 min. Alternative lever responses resulted in the illumination of its associated visual-cue for 20 s, the deactivation of the house light, and the retraction of both levers for 5 min.

Withdrawal (20 days). Rats were treated as described in Experiment 1, with the exception that they were matched on BW, the number of active and alternative paired cue presentations during the last five days of training.

Heroin/Non-heroin visual-cue-seeking test (2 days). On W15 and W20, rats were returned to the operant conditioning chambers for a 3 h test under extinction conditions. Rats were tested under two separate, counterbalanced protocols. ***Protocol 1:*** Lever responses were reinforced under a FR1+5 min ITI schedule of reinforcement, as described in phase 3 of the self-administration training. ***Protocol 2:*** Rats were treated similarly to those in test 1 except that the lever responses were reinforced under a FR3+5 min ITI, schedule of reinforcement. See **Figure 4.6A** for a summary of the experimental procedure.

4.3.6 Statistical Analyses

All analyses were conducted using Prism software (Graphpad Software, version 6.0), however CIs around the effect sizes were calculated using R (The R Project for Statistical Computing, version 3.3.1). The critical threshold for statistically significant results was set at $p \leq 0.05$ and adjusted for multiple comparisons using the Holm-Sidak correction when necessary. All statistically significant interactions were investigated with *post-hoc* tests applying the Holm-Sidak correction. All *t*-tests were followed by Cohen's *d* effect size calculations, while all ANOVAs were followed by eta-squared effect size calculations. All CIs reported were calculated around their respective effect sizes.

Experiment 1. Planned comparisons were used to analyze breakpoints, active and alternative lever responses made under a PR schedule. Three independent samples *t*-tests were conducted, with *feeding condition* (Sated, FDR) as the independent variable were conducted.

Experiment 2. Planned comparisons were used to analyze the number of cue presentations, and active minus alternative ('active - alternative') nose poke responses made during the test session. Active and alternative nose poke responses were analyzed using a mixed design ANOVA, with *feeding condition* (Sated, FDR) serving as the between-subjects factor and *aperture type* (Alternative, Active) as the within-subjects factor. Three ANOVAs were conducted, one for each test day (W15, W18, and W23). The Holm-Sidak method was used to correct for multiple comparisons when appropriate.

Experiment 3. The number of cue presentations, active, and alternative lever responses were analyzed using separate mixed design ANOVAs, with *feeding condition* (Sated, FDR) serving as the between subjects factor, and *lever type* (Alternative, Active) as the within-subjects factor.

Experiment 4. Planned comparisons were used to analyze the number of cue presentations and 'active - alternative' nose poke responses during each test session. Separate mixed design ANOVAs (one for each test day; W15 and W18), with *feeding condition* (Sated, FDR) as the between-subjects factor and *aperture type* (Alternative, Active) serving as the within-subjects

factor, were used to analyze the differences in active and alternative (*4A:* visual/auditory-cue-paired, *4B:* auditory-cue-paired) nose pokes. When appropriate, the Holm-Sidak method was used to correct for multiple comparisons.

Experiment 5. The number of active and alternative responses, and cue presentations on test days were analyzed using a two-way mixed design ANOVA with *feeding condition* (Sated, FDR) as the between-subjects factor and *lever type* (Active, Alternative) serving as the within-subjects factor.

For a summary of active and alternative lever/nose poke device pairings, across experiments, see **Table 4.1**.

	Active Lever	Alternative Lever
Exp.1	heroin+visual/auditory-cue-	non-paired
Exp.2	heroin+visual/auditory-cue-	non-paired
Exp. 3	visual/auditory-cue-paired	non-paired
Exp. 4A	heroin+visual/auditory-cue-	visual/auditory-cue-paired
Exp. 4B	heroin+auditory-cue-paired	auditory-cue-paired
Exp. 5	heroin+visual-cue-paired	visual-cue-paired

Table 4.1. Summary presentation of the active and alternative lever/nose poke pairings for each experiment.

4.4 RESULTS

All rats acquired reliable heroin self-administration behaviour. For each experiment, mean ± SEM number of infusions and lever responses made on the last day of heroin self-administration

training are shown in **Table 4.2**. There were no statistically significant differences in any of the reported parameters between the different experimental groups within each experiment.

	Infusions or Active Cue	Alternative Cue	Active Lever	Alternative Lever
Exp. 1	36.49±2.45	–	129.57±18.79	13.20±3.42
Exp. 2	37.85±2.51	–	102.30±9.86	7.45±1.66
Exp. 3	14.17±1.69	–	28.48±3.98	28.83±8.18
Exp. 4A	42.59±4.06	12.76±2.64	135.18±24.41	24.18±5.25
Exp. 4B	49.63±5.85	8.75±1.62	191.13±42.59	24.63±4.37
Exp. 5	23.61±2.46	6.17±1.07	23.61±2.46	6.17±1.07

Table 4.2. Mean±SEM number of heroin infusions/active cue presentations, alternative cue presentations, and active and alternative lever responses made on the last day of training (9 h) for each experiment.

Experiment 1: The effects of chronic food restriction on lever pressing reinforced by heroin-associated cues under a progressive ratio schedule of reinforcement.

Five rats were removed due to a failure to train, being an outlier during training (> 4 SD), catheter leakage, a detached head cap, and a technical error during testing. Therefore, the final analyses were conducted on 35 rats in two experimental groups: Sated (n = 18), FDR (n = 17). See **Table 4.3** for average BWs on test day.

Initially, rats were tested under a PR 20 s timeout schedule of reinforcement, where responses during the timeout period were recorded but did not contribute to the progression of the reinforcement schedule. To test the impact of non-reinforced responses during the timeout period, a second set of rats were tested under a PR 5 s timeout. Given that there were no differences in the breakpoint levels reached under both schedules, the data were combined within each feeding condition (Sated, FDR).

	Sated - Body Weight	FDR - Body Weight	*t*-test
Exp. 1			
W15	419.83±8.75	312.76±5.58	$t_{(33)} = 10.19$, $p < .001$, $d = 3.45$, $CI = [2.38, 4.50]$
Exp. 2			
W15	433.87±4.68	314.42±4.58	$t_{(45)} = 17.31$, $p < .001$, $d = 5.05$, $CI = [3.86, 6.23]$
W18	447.70±5.54	312.17±4.75	$t_{(44)} = 18.57$, $p < .001$, $d = 5.48$, $CI = [4.19, 6.75]$
W23	468.48±5.83	313.09±4.92	$t_{(43)} = 20.22$, $p < .001$, $d = 6.03$, $CI = [4.62, 7.42]$
Exp. 3			
W15	447.93±8.81	367.71±5.50	$t_{(27)} = 7.60$, $p < .001$, $d = 3.49$, $CI = [2.00, 4.94]$
Exp. 4A			
W15	458.50±6.85	333.67±5.46	$t_{(15)} = 14.40$, $p < .001$, $d = 7.00$, $CI = [4.33, 9.64]$
W18	471.00±6.70	328.00±4.71	$t_{(15)} = 17.78$, $p < .001$, $d = 8.64$, $CI = [5.42, 11.83]$
Exp. 4B			
W15	466.75±12.32	334.14±13.60	$t_{(13)} = 7.24$, $p < .001$, $d = 3.75$, $CI = [1.97, 5.47]$
W18	483.00±14.44	328.86±13.62	$t_{(11)} = 10.25$, $p < .001$, $d = 5.70$, $CI = [3.09, 8.27]$
Exp. 5			
W15	475.50±12.72	370.60±6.54	$t_{(16)} = 7.78$, $p < .001$, $d = 3.69$, $CI = [2.10, 5.24]$
W20	492.13±14.39	365.60±7.00	$t_{(16)} = 8.43$, $p < .001$, $d = 4.00$, $CI = [2.32, 5.64]$

Table 4.3. Mean±SEM average body weights (BW) on test day for Sated and food-restricted (FDR) rats. *T*-tests, p values, effect sizes, and confidence intervals for each effect size are reported.

Planned comparisons did not reveal any statistically significant differences between feeding conditions (Sated, FDR) for the number of breakpoints (Sated: 10.57±0.66, FDR: 11.59±0.85; $t_{(33)} = 0.97$, $p = .342$, $d = 0.33$, $CI = [-0.34, 0.99]$), active ($t_{(33)} = 1.22$, $p = .233$, $d = 0.41$, $CI = [-0.26, 1.08]$), or alternative ($t_{(33)} = 0.88$, $p = .383$, $d = 0.30$, $CI = [-0.37, 0.96]$) lever responses during the test session (**Figure 4.1B**).

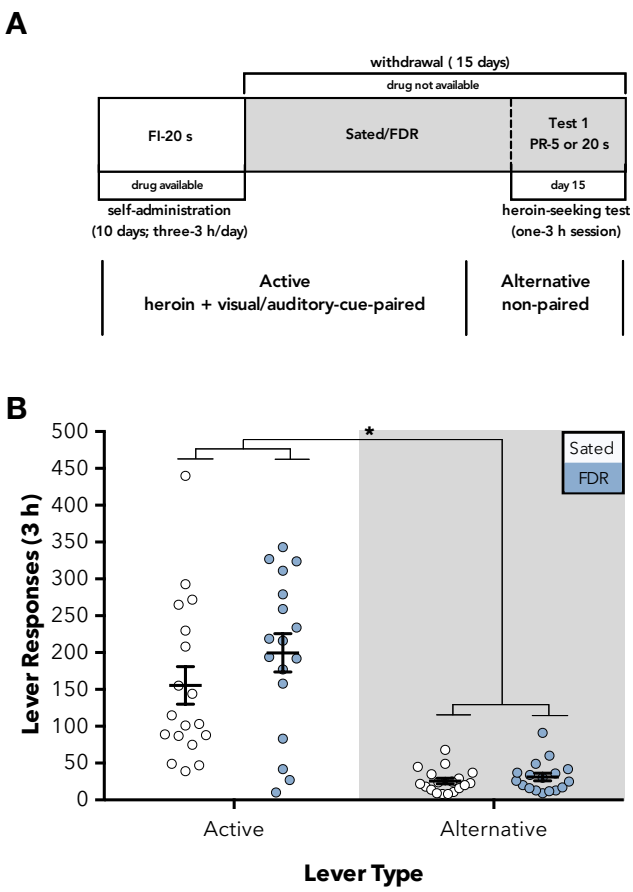

Figure 4.1. Chronic food restriction did not change lever responding reinforced by heroin-associated cues under a progressive ratio (PR) schedule of reinforcement. (**A**) Timeline and design of Experiment 1. Rats were trained to self-administer heroin under a fixed interval 20 s (FI-20) schedule of reinforcement. Active lever responses were reinforced with heroin infusions (0.1 mg/kg/infusion), paired with a visual/auditory cue. Responses on the alternative lever had no programmed consequences. Next, rats underwent 15 days of withdrawal under food restriction (FDR; n = 17) or sated (n = 18) conditions. Finally, rats underwent a 3 h heroin-seeking test under extinction conditions and a PR schedule of reinforcement. (**B**) The number (M±SEM) of active and alternative lever responses. *p < .001.

Experiment 2: The effects of chronic food restriction on acquisition of a novel nose poke response reinforced solely by heroin-associated cues

Three rats were removed from the analyses due to failure to train (n = 2) or unusually high active lever responses during training (> 3 SD). The final analyses on W15 were conducted on 47 rats across two experimental groups: Sated (n = 23) and FDR (n = 24). On W18, the analyses included 46 rats, as one rat in the FDR group (n = 23) was removed due to a detached head cap. The analyses on W23 was conducted on a total of 45 rats due to another detached head cap in the FDR group (n = 22). See **Table 4.3** for average BWs on test days.

Across all test sessions, planned comparisons revealed that food-restricted rats had a statistically significant preference for responses in the active aperture, reflected by a higher aperture type difference score ('active - alternative' nose pokes) compared to sated rats (W15: $t_{(45)} = 6.37$, $p < .001$, $d = 1.86$, $CI = [1.16, 2.54]$; W18: $t_{(44)} = 4.98$, $p < .001$, $d = 1.47$, $CI = [0.81, 2.12]$; W23: $t_{(43)} = 4.57$, $p < .001$, $d = 1.36$, $CI = [0.71, 2.01]$; **Figure 4.2B**). Furthermore, a statistically significant increase in the number of cue presentations earned by the FDR group, versus the Sated group, was observed (W15: $t_{(45)} = 6.33$, $p < .001$, $d = 1.85$, $CI = [1.15, 2.52]$; W18: $t_{(44)} = 5.35$, $p < .001$, $d = 1.58$, $CI = [0.90, 2.23]$; W23: $t_{(43)} = 5.44$, $p < .001$, $d = 1.62$, $CI = [0.94, 2.29]$; **Figure 4.2C**).

ANOVAs performed on the number of nose pokes revealed a statistically significant, food-restriction-induced increase across all three test sessions (*feeding condition*; W15: $F_{(1,45)} = 16.77$, $p < .001$, $\eta^2 = 0.38$, $CI = [0.10, 0.42]$; W18: $F_{(1,44)} = 11.25$, $p = .002$, $\eta^2 = 0.21$, $CI = [0.05, 0.36]$; W23: $F_{(1,43)} = 5.95$, $p = .023$, $\eta^2 = 0.13$, $CI = [0.01, 0.27]$). Overall, a greater number of nose-pokes into the active, compared to the alternative, aperture were made during all test sessions (*aperture type;* W15: $F_{(1,45)} = 110.30$, $p < .001$, $\eta^2 = 0.71$, $CI = [0.58, 0.78]$; W18: $F_{(1,44)} = 42.54$, $p < .001$, $\eta^2 = 0.49$, $CI = [0.30, 0.61]$; W23: $F_{(1,43)} = 20.78$, $p < .001$, $\eta^2 = 0.49$, $CI = [0.14, 0.47]$).

A stronger impact of food restriction on nose pokes into the active module was supported by a statistically significant *feeding condition X aperture type interaction* (W15: $F_{(1,45)} = 40.58$, $p < .001$, $\eta^2 = 0.47$, $CI = [0.29, 0.60]$; W18: $F_{(1,44)} = 24.80$, $p < .001$, $\eta^2 = 0.36$, $CI = [0.17, 0.50]$; W23: $F_{(1,43)} = 20.84$, $p < .001$, $\eta^2 = 0.33$, $CI = [0.14, 0.47]$). When analyzing the number of nose-pokes into the active and alternative aperture separately, a statistically significant, food-restriction-induced augmentation of nose-pokes made into the active (W15: $t_{(45)} = 5.57$, $p < .001$, $d = 1.63$, $CI = [0.96, 2.28]$; W18: $t_{(44)} = 4.76$, $p < .001$, $d = 1.50$, $CI = [0.75, 2.04]$; W23: $t_{(43)} = 4.14$, $p < .001$, $d = 1.62$, $CI = [0.94, 2.29]$; **Figure 4.2D-F**), but not the alternative aperture (W15: $t_{(45)} = 1.20$, $p = .236$, $d = 0.35$, $CI = [0.23, 0.92]$; W18: $t_{(44)} = 1.54$, $p = .130$, $d = 0.45$, $CI = [-0.13, 1.04]$;

W23: $t_{(43)}$ = 1.81, p = .070, d = 0.54, CI = [-0.06, 1.13]) was observed across all three test sessions, compared to sated rats.

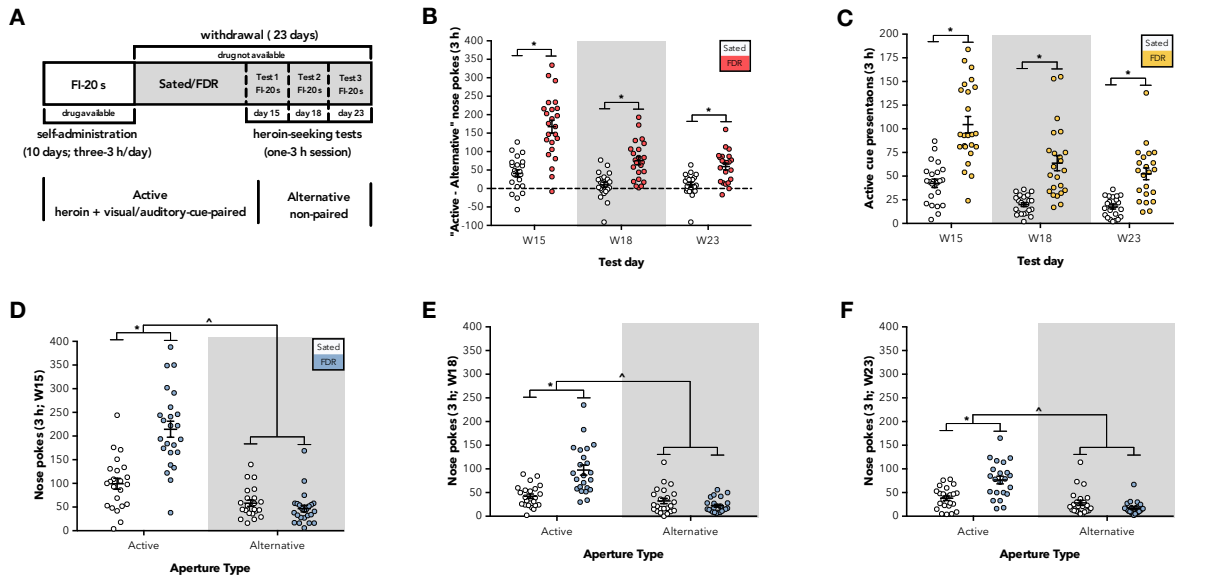

Figure 4.2. Chronically food-restricted rats acquired a heroin-cue-driven, novel operant response at greater levels than sated rats. All data are presented as mean ±SEM. Analyses were conducted on two experimental groups: Sated (W15, W18, W23: n = 23) and food-restricted (FDR; W15: n = 24; W18: n = 23; W23: n = 22). (**A**) Timeline and design of Experiment 2. Following heroin self-administration training, rats underwent 15 days of withdrawal (W) under FDR or sated conditions. Next, rats underwent a test for the acquisition of a novel response under extinction conditions, where levers were replaced with nose poke devices. Nose pokes in the active aperture were reinforced by heroin-associated cues, while nose pokes in the alternative aperture had no programmed consequences. (**B**) The number of 'active - alternative' nose pokes during testing on W15, W18, and W23. (**C**) The number of heroin-paired cue presentations earned on test days. (**D-F**) The number of nose poke responses made on W15, W18, and W23, in the active and alternative apertures. ***,**^p < .001.

Food-restricted rats consistently demonstrated a greater number of nose-pokes into the active compared to the alternative aperture across testing sessions (W15: $t_{(45)}$ = 12.10, p < .001, d = 3.50, CI = [2.57, 4.39]; W18: $t_{(44)}$ = 8.13, p < .001, d = 2.40, CI = [1.63, 3.15]; W23: $t_{(43)}$ = 7.66, p < .001, d = 2.31, CI = [1.53, 3.07]). Rats in the Sated group exhibited a similar, statistically significant difference, however only during the first test session (W15: $t_{(45)}$ = 2.89, p = .012, d = 0.85, CI = [0.24, 1.45]; W18: $t_{(44)}$ = 1.09, p = .484, d = 0.32, CI = [-0.26, 0.90]; W23: $t_{(43)}$ = 1.30, p = .360, d = 0.38, CI = [-0.20, 0.96]).

Experiment 3: The effects of chronic food restriction on instrumental responses reinforced by a compound visual/auditory cue.

One rat from the FDR group was removed from all analyses due to abnormally high (> 3 SD) active lever responses on test day. The final analyses were conducted on 29 rats in two experimental groups: Sated (n = 15) and FDR (n = 14). See **Table 3** for average BWs on test day.

Overall, the number of active lever responses were statistically significantly higher than alternative lever responses (*lever type* - $F_{(1,27)}$ = 13.51, p = .001, η^2 = 0.33, *CI* = [0.10, 0.51]) across feeding groups. No statistically significant differences in lever responding were observed between the FDR and Sated groups (*feeding condition* - $F_{(1,27)}$ = 0.37, p = .550, η^2 = 0.03, *CI* = [0.00, 0.15]; *feeding condition x lever type* - $F_{(1,27)}$ = 0.29, p = .597, η^2 = 0.01, *CI* = [0.00, 0.14]). No statistically significant effect of feeding condition on the number of cue presentations was observed (Sated: 27.60±3.07, FDR: 31.64±3.95; $t_{(27)}$ = .82, p = .422, d = 0.30, *CI* = [-0.43, 1.03]; **Figure 4.3A**).

Experiment 4: The effects of chronic food restriction on acquisition of a novel nose poke response reinforced by heroin-paired or neutral cues

Experiment 4A. Two rats were removed from the analyses due to catheter leakage and a detached head cap. Therefore, analyses on W15 and W18 were conducted on 17 rats across two experimental groups: Sated (n = 8) and FDR (n = 9). See **Table 3** for average BWs on test days.

Compared to sated rats, a strong trend for higher aperture-type difference score ('active - alternative' nose pokes) was observed in the FDR rats on the W15 test, with a large effect size (W15: $t_{(15)}$ = 2.07, p = .056, d = 1.01, *CI* = [-0.03, 2.01]; W18: $t_{(15)}$ = 0.80, p = .438, d = 0.39, *CI* = [-0.58, 1.34]; **Figure 4.4B**).

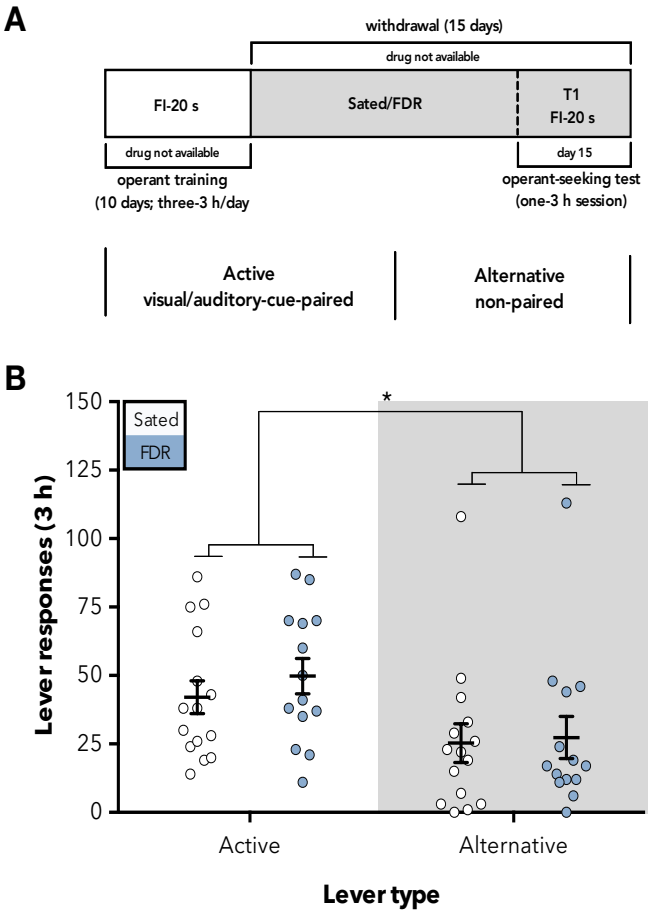

Figure 4.3. Chronic food restriction did not change lever responses reinforced by a visual/auditory compound stimulus. All data are presented as mean ±SEM. Analyses were conducted on two experimental groups: Sated (n = 15) and food-restricted (FDR; n = 14). (**A**) Timeline and design of Experiment 3. Following operant conditioning reinforced by a visual/auditory compound stimulus, rats underwent 15 days of "withdrawal" under FDR or sated conditions. Next, rats were tested in one 3 h session where active (visual/auditory-cue-paired) and alternative (no programmed consequences) lever contingencies were identical to the operant-conditioning phase. (**B**) Active and alternative lever responses made on test day. ***p* = .001.

Planned comparisons indicated that FDR rats received statistically significantly more active cue presentations, compared to sated rats on the first test session, with a strong trend in the second session (W15: $t_{(15)}$ = 2.52, p = .024, d = 1.22, CI = [0.16, 2.25]; W18: $t_{(15)}$ = 2.47, p = .026 (*ns* following correction for multiple comparisons), d = 1.20, CI = [0.14, 2.23]; **Figure 4.4C**). In addition, a statistically significant increase in the number of alternative cue presentations earned by the FDR, compared to sated rats, was observed on W18 ($t_{(15)}$ = 2.60, p = .020, d = 1.26, CI = [0.19, 2.30]), but not W15 ($t_{(15)}$ = 1.93, p = .073, d = 0.94, CI = [-0.08, 1.93]; **Figure 4.4D**).

On W15 and W18, a higher number of nose poke responses aimed at the active, compared to the alternative aperture was supported by a statistically significant effect of *aperture type* (W15:

$F_{(1,15)}$ = 13.40, p = .002, η^2 = 0.47, *CI* = [0.14, 0.65]; W18: $F_{(1,15)}$ = 7.01, p = .018, η^2 = 0.32, *CI* = [0.03, 0.54]; **Figure 4.4E-F**). A statistically significant, food-restriction-induced augmentation of nose poke responses was also observed (*feeding condition* - W15: $F_{(1,15)}$ = 4.50, p = .051, η^2 = 0.33, *CI* = [0.00, 0.47]; W18: $F_{(1,15)}$ = 7.14, p = .017, η^2 = 0.36, *CI* = [0.03, 0.54]), which was non-selective for aperture type. The *feeding condition X aperture type* interaction was not statistically significant (W15: $F_{(1,15)}$ = 4.29, p = .056, η^2 = 0.22, *CI* = [0.00, 0.46]; W18: $F_{(1,15)}$ = 0.64, p = .438, η^2 = 0.04, *CI* = [0.00, 0.26]). However, upon both visual inspection of the data and effect size analyses, it is likely that augmented active versus alternative aperture responses were largely driven by the food-restricted rats (W15: d = 1.50, W18: d = 0.91) (Sated: W15: d = 0.89, W18: d = 1.02) at least in the first test session. Planned comparisons revealed that the FDR rats performed statistically significantly more active nose pokes compared to sated rats on the first test day (W15: $t_{(15)}$ = 2.35, p = .033, d = 1.14, *CI* = [0.09, 2.16]), but not on the second test day (W18: $t_{(15)}$ = 2.17, p = .104, d = 1.05, *CI* = [0.02, 2.06]; **Figure 4.4E-F**).

Across both test sessions, no statistically significant differences were observed in the number of alternative nose poke responses between the FDR and sated rats (W15: $t_{(15)}$ = 0.61, p = .548, d = 0.30, *CI* = [-0.67, 1.25]; W18: $t_{(15)}$ = 1.73, p = .104, d = 0.84, *CI* = [-0.17, 1.83]; **Figure 4.4E-F**).

Experiment 4B (auditory cue). Prior to test, four rats were removed from all analyses due to detached head caps. Following the first test session (W15), an additional rat was removed also due to a detached head cap. Analyses on W15 and W18 were conducted on 14-15 rats across two experimental groups: Sated (W15: n = 8, W18: n = 7) and FDR (W15: n = 7, W18: n = 7). See **Table 4.3** for average BWs on test days.

A statistically significant higher aperture type difference score was observed in the FDR group compared to the Sated group during both test sessions, indicating a strong preference for responding into the active versus alternative aperture in the FDR rats (W15: $t_{(13)}$ = 4.67, p <

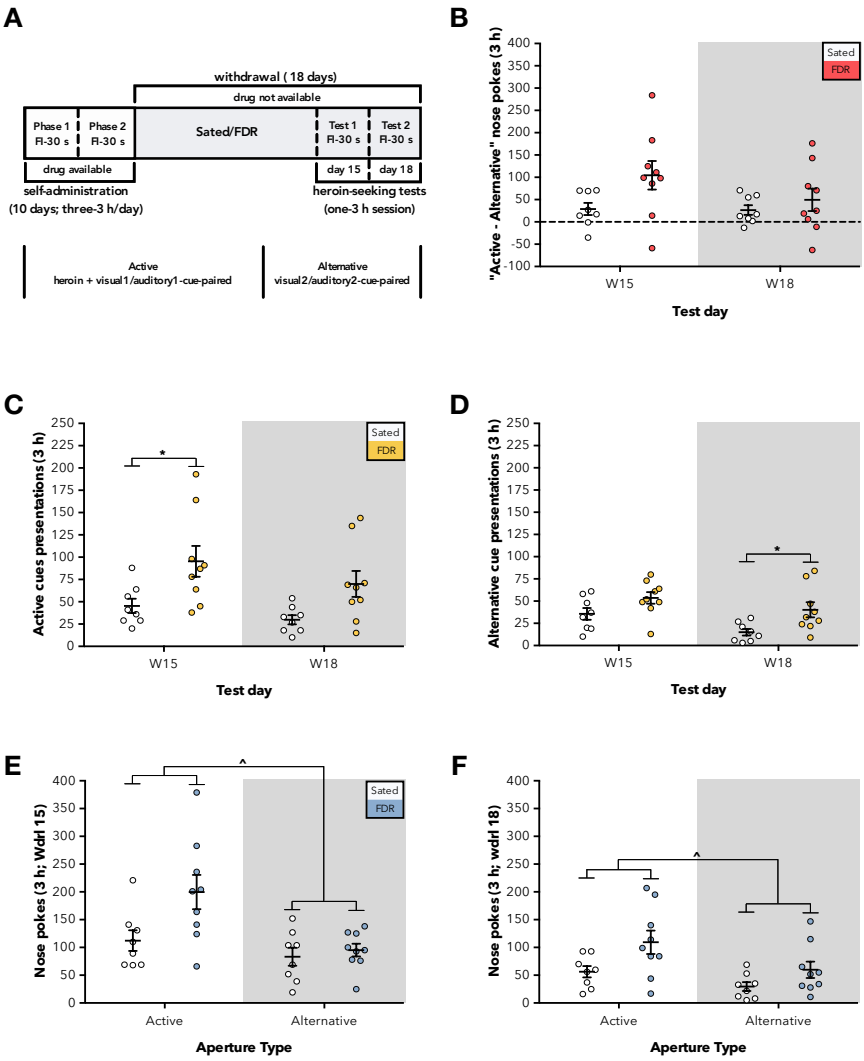

Figure 4.4. Presentation of an alternative lever paired with a visual/auditory neutral cue slightly attenuated the food-restriction-induced augmentation of acquisition of a novel nose poke response reinforced by heroin-associated cues. All data are presented as mean ±SEM. Analyses were conducted on two experimental groups: Sated (*n* = 8) and food-restricted (FDR; *n* = 9). **(A)** Timeline and design of Experiment 4A. Rats were trained to self-administer heroin in two phases. *Phase 1 (day 1-5):* Responses on the active lever (heroin+visual/auditory-cue-paired) were reinforced under a fixed interval 30 s (FI-30) schedules of reinforcement; responses on the alternative lever had no programmed consequences. *Phase 2 (day 6-10):* The alternative lever contingency was altered to support a neutral visual/auditory compound stimulus activated under a FI-30 s schedule of reinforcement. Next, rats underwent 15 days of withdrawal (W) under sated or FDR conditions. Rats then underwent two tests to measure the acquisition of a novel response (W15, W18) under extinction conditions, where levers were replaced with nose poke devices. Responses in the active and alternative apertures activated the previously paired heroin-associated or neutral cues, respectively. **(B)** The number of "active - alternative" nose pokes during testing on W15 and W18. **(C-D)** The number of active- or alternative-paired cue presentations earned on both test days. **(E-F)** The number of nose poke responses made on W15 and W19, respectively. ***,^***p* < .05.

.001, d = 2.42, *CI* = [1.02, 3.76]; W18: $t_{(12)}$ = 2.63, p = .022, d = 1.41, *CI* = [0.20, 2.57]; **Figure 4.5B**).

On both test sessions, an increase in nose pokes targeted toward the active aperture was observed, as per the main effect of *aperture type* (W15: $F_{(1,13)}$ = 64.31, p < .001, η^2 = 0.83, *CI* = [0.61, 0.89]; W18: $F_{(1,12)}$ = 47.70, p < .001, η^2 = 0.80, *CI* = [0.53, 0.87]). In both test sessions, FDR rats showed a greater overall number of nose poke responses compared to sated rats (*feeding condition* - W15: $F_{(1,13)}$ = 16.83, p = .001, η^2 = 0.69, *CI* = [0.20, 0.72]; W18: $F_{(1,12)}$ = 5.89, p = .032, η^2 = 0.30, *CI* = [0.02, 0.56]); an effect that was driven by the responses into the active aperture (*feeding condition X aperture type* - W15: $F_{(1,13)}$ = 16.98, p = .001, η^2 = 0.57, *CI* = [0.20, 0.72]; W18: $F_{(1,15)}$ = 6.92, p = .022, η^2 = 0.37, *CI* = [0.03, 0.59]). Although higher responses into the active aperture was statistically significant for both feeding conditions, the effect size for the calculated difference was considerably larger in FDR rats (FDR [active vs. alternative]: $t_{(13)}$ = 8.31, p < .001, d = 4.16, *CI* = [2.32, 5.94]; Sated (active vs. alternative): $t_{(13)}$ = 2.85, p = .014, d = 1.52, *CI* = [0.29, 2.71]). Furthermore, FDR rats demonstrated a statistically significant augmentation of active nose-poking behaviour compared to sated rats (W15: $t_{(13)}$ = 4.34, p < .001, d = 2.25, *CI* = [0.89, 3.55]; W18: $t_{(13)}$ = 5.40, p < .001, d = 2.79, *CI* = [1.30, 4.24]; **Figure 4.5E-F**), which was accompanied by a statistically significant increase in active cue presentations (W15: $t_{(13)}$ = 5.40, p < .001, d = 2.79, *CI* = [1.30, 4.24]; W18: $t_{(12)}$ = 3.29, p = .006, d = 1.76, *CI* = [0.48, 2.99]; **Figure 4.5C**). No statistically significant differences between feeding conditions were observed for alternative nose pokes (W15: $t_{(13)}$ = 1.56, p = .143, d = 0.81, *CI* = [-2.67, 1.85]; W18: $t_{(12)}$ = 0.14, p = .891, d = 0.07, *CI* = [-0.97, 1.12]; **Figure 4.5E-F**) or the alternative cue presentations (W15: $t_{(13)}$ = 2.50, p = .027 (*ns* following correction for multiple comparisons), d = 1.29, *CI* = [0.15, 2.40]; W18: $t_{(12)}$ = 0.32, p = .751, d = 0.17, *CI* = [-0.88, 1.22]; **Figure 4.5D**).

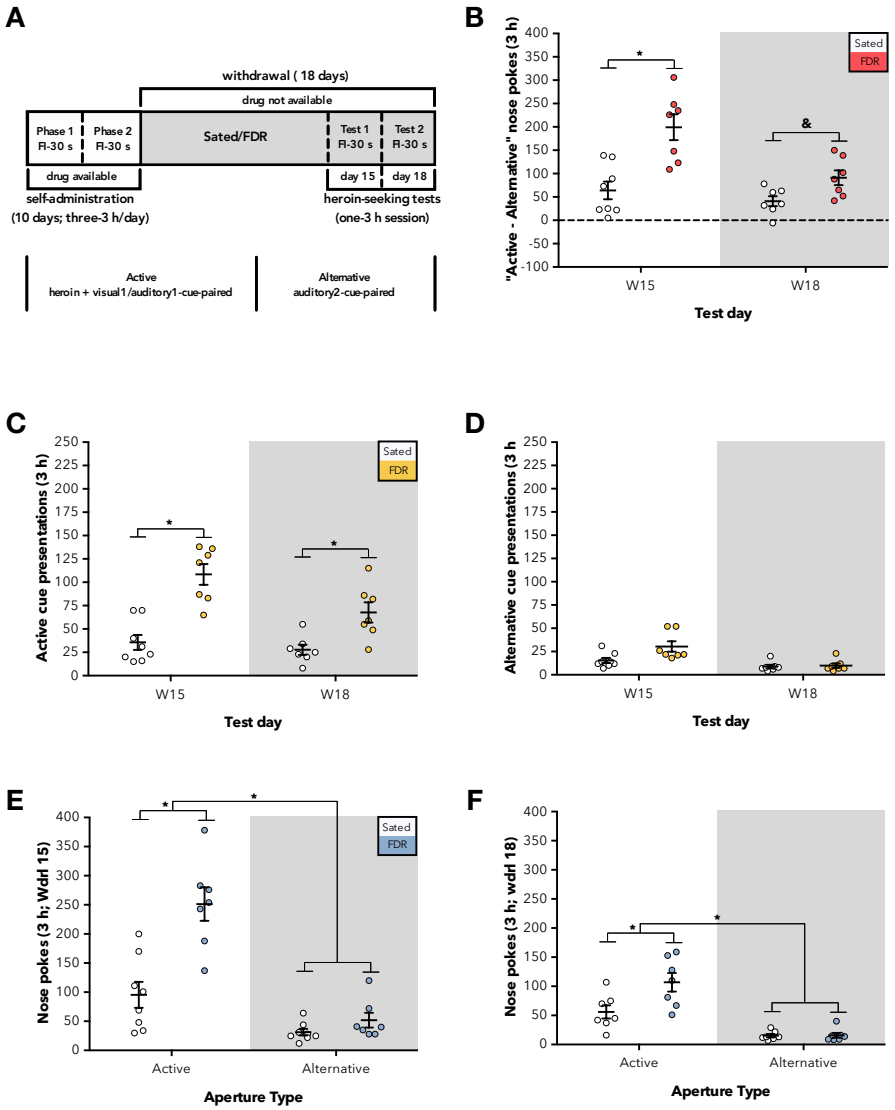

Figure 4.5. Presentation of an alternative lever paired with an auditory neutral-cue did not interfere with the food-restriction-induced augmentation of the acquisition of a novel nose poke response reinforced by heroin-associated cues. All data are presented as mean ± SEM. Analyses were conducted on two experimental groups: Sated (W15: *n* = 8, W18: *n* = 7) and food-restricted (FDR; W15: *n* = 7, W18: *n* = 7). (**A**) Rats were trained, manipulated and tested as described in Figure 4, except that the neutral stimulus paired with the alternative lever and aperture was auditory. (**B**) The number of 'active - alternative' nose pokes during testing on W15 and W18. (**C-D**) The number of active- or alternative-paired cue presentations earned on both test days. (**E-F**) The number of nose poke responses made on W15 and W18. $^{*}p < .001$, $^{\&}p < .05$.

Experiment 5: The effects of chronic food restriction on the choice between heroin-paired cues and neutral cues.

Two rats were removed from the analyses due to a failure to train and catheter leakage. Thus, analyses were conducted on a total of 18 rats in two experimental groups: Sated (n = 8) and FDR (n = 10). See **Table 4.3** for average BWs on test days.

Training data are presented in **Figure 4.6B**. The number of lever responses during the "active days" (heroin-paired) under a FI-20 s schedule almost doubled those performed during the "alternative days" (Figure 4.6B, left panel). Interestingly, following the switch to the FR1+5 min ITI schedule, rats received a similar number of active cue presentations, heroin infusions, alternative-cue presentations as during the FI-20 s phase (Figure 4.6B, middle panel). Moreover, a similar pattern continued during the choice phase (Figure 4.6B, right panel).

FR1+5 min ITI: On test day, a statistically significant main effect of *lever type* supports a higher overall number of responses on the active (18.28±1.28), compared to the alternative (6.22±0.62) lever ($F_{(1,16)}$ = 62.71, p < .001, η^2 = 0.68, CI = [0.58, 0.86]). No statistically significant main effect of *feeding condition* ($F_{(1,16)}$ = 1.42, p = .251, η^2 = 0.01, CI = [0.00, 0.31]) nor *feeding condition X lever type* interaction ($F_{(1,16)}$ = 0.49, p = .495, η^2 = 0.005, CI = [0.00, 0.23]) was observed.

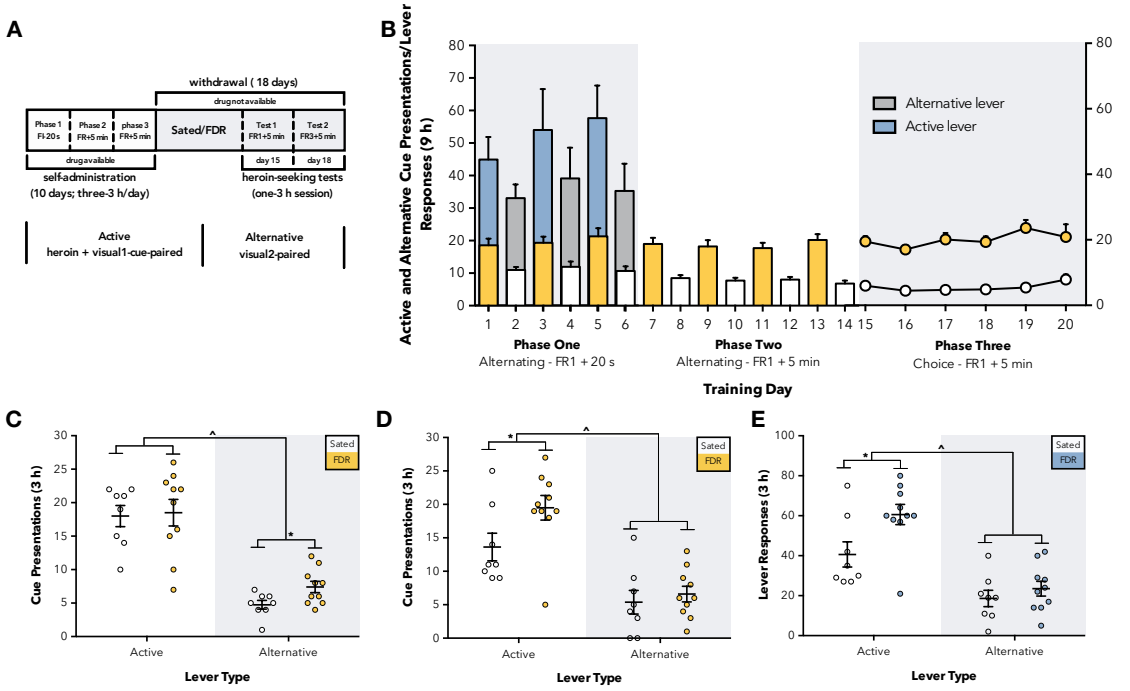

Figure 4.6. Chronic food restriction increased responding on a lever reinforced by heroin-associated cue, under a choice procedure. All data are presented as mean ± SEM. Analyses were conducted on two experimental groups: Sated (n = 8) and food-restricted (FDR; n = 10). (**A**) Timeline and design of Experiment 4B. Rats were trained to self-administer heroin in three phases. *Phase 1:* rats had alternating access to an active (heroin+visual-cue1-paired) or alternative (visual-cue2-paired) lever under a fixed ratio 20 s (FI-20 s) schedule of reinforcement. *Phase 2:* The reinforcement schedule was changed to fixed ratio 1+ 5 min inter-trial-interval (FR1+5 min ITI) *Phase 3:* The two levers, under a FR1+5 min ITI were simultaneously available. Next, rats underwent 20 days of withdrawal (W) with unrestricted (Sated) or restricted (FDR) access to chow. On W15 and W20, rats underwent a 3 h heroin-seeking test under extinction conditions, with FR1+5 min ITI or FR3+5 min ITI schedule of reinforcement, respectively. (**B**) The number of active and alternative lever responses and cue presentations earned in each phase. (**C**) The number of active and alternative cue presentations during testing with FR1+5 min ITI schedule of reinforcement (W15). (**D-E**) The number of active and alternative cue presentations and lever responses during testing with FR3+5 min ITI schedule of reinforcement (W20), respectively. sessions. *****p < .05, **^**p < .001.

Compared to rats in the Sated group, planned comparisons identified a statistically significant augmentation in the FDR rats for alternative ($t_{(16)}$ = 2.38, p = .030, d = 1.13, CI = [0.11, 2.12]) but not active cue presentations ($t_{(16)}$ = 0.19, p = .852, d = 0.09, CI = [-0.84, 1.02]). The number of lever responses is identical to the number of cue presentations given the retraction of levers following each response (**Figure 4.6C**).

FR3+5 min ITI: On test day, a statistically significant main effect of *lever type* supports the overall higher number of responses on the active (51.72±4.53), compared to the alternative (21.33±2.73) lever ($F_{(1,16)}$ = 31.96, p < .001, η^2 = 0.46, *CI* = [0.37, 0.78]; **Figure 4.6E**). In the FDR group, a statistically significant augmentation in overall lever responding (42.05±5.23) compared to the Sated group (29.63±4.60) was observed (*feeding condition*: $F_{(1,16)}$ = 7.87, p = .013, η^2 = 0.08, *CI* = [0.05, 0.54]). The *feeding condition X lever type* interaction was not statistically significant ($F_{(1,16)}$ = 2.09, p = .168, η^2 = 0.03, *CI* = [0.00, 0.35]).

A statistically significant main effect of *lever type* ($F_{(1,16)}$ = 33.54, p < .001, η^2 = 0.48, *CI* = [0.39, 0.78]) revealed that more active lever-associated cues (16.89±1.51) were earned compared to alternative cues (6.06±0.99).. An increase in cue presentations was also observed for the FDR group (13.05±1.82) compared to the stated group (9.50±1.69; *feeding condition*: $F_{(1,16)}$ = 4.89, p = .042, η^2 = 0.19, *CI* = [0.005, 0.46]). The *feeding X lever type* interaction was not statistically significant ($F_{(1,16)}$ = 1.62, p = .221, η^2 = 0.02, *CI* = [0.00, 0.32]). Planned comparisons found a statistically significant increase in the number of active cue presentations ($t_{(16)}$ = 2.12, p = .049, d = 1.01, *CI* = [0.00004, 1.98]; **Figure 4.6D**) and active lever responses ($t_{(16)}$ = 2.51, p = .023, d = 1.19, *CI* = [0.16, 2.19]; **Figure 4.6E**) performed by the FDR versus the Sated group. No statistically significant differences were observed in alternative cue presentations ($t_{(16)}$ = 0.60, p = .557, d = 0.28, *CI* = [-0.65, 1.22]) or alternative lever responses ($t_{(16)}$ = 0.88, p = .391, d = 0.42, *CI* = [-0.53, 1.35]).

4.5 DISCUSSION

Visual stimuli are commonly used to measure the effects of cue-induced drug seeking. However, the inherent motivational properties of visual stimuli are often ignored by the literature, making the underlying nature of seeking behaviour difficult to resolve. As such, the present study was designed to examine the motivational properties of heroin-associated cues and non-drug-paired visual stimuli. Here, we highlight the necessity for a more judicious

approach when using visual stimuli to study drug-seeking behaviour, in both food-restricted, and to a greater degree, *ad libitum* fed rats. Initially, we observed an overall increase in active nose poke responses (reinforced by visual/auditory cues previously paired with heroin), and a more targeted acquisition of a novel nose poke responses (active vs. alternative responses) in food-restricted, compared to sated rats (Experiment 2). When presented with a visual/auditory cue-paired alternative stimulus (Experiment 4), the expected augmentation in active nose pokes compared to alternative responses was absent in the sated rats. Within the food-restricted group, the increase in active nose pokes, compared to alternative responses, first remained present but later diminished. This may be due to the potential discord between the incentive motivational properties of the visual stimulus upon its pairing with the alternative responses (Experiment 4A). In contrast, when a tone was paired to the alternative nose poke, the enhanced acquisition of active responses (reinforced by visual/auditory cues previously paired with heroin) in food-restricted rats re-emerged (Experiment 4B) to levels similar to those observed when no stimulus was paired to the alternative nose poke (Experiment 2).

These data were further supported in a choice procedure between a heroin-paired or an alternative light (FR1+5 min ITI), where food-restricted and sated rats responded equally for both cues. Interestingly, this similarity in choice behaviour was not observed under an FR3+5 min ITI schedule of reinforcement which required a greater commitment in choice. As a result of this increased commitment, food-restricted rats exhibited an augmentation in responding compared to their sated counterparts, as well as a greater level of responses on the active versus alternative lever (Experiment 5). Collectively, these data imply that drug-seeking behaviour may be difficult to separate from the inherent incentive motivational properties of visual stimuli. This may be especially true given that drug-seeking behaviour is commonly studied using simple schedules of reinforcement, and at least in our case, in food-restricted and sated rats.

The progressive ratio conundrum and the need for an alternate procedure to evaluate the incentive motivational properties of heroin cues (Experiment 1; Figure 1). When using a

PR schedule, responding is almost exclusively performed under conditions in which the primary reinforcer (e.g., food, drug) is available. As a result, data collected on responses which are solely reinforced by drug-paired cues remain sparse. In the case of a natural reward, Chaudhri et al. (2006a) reported that a sucrose-paired stimulus supported responding at greater levels than a sucrose-unpaired stimulus, under a PR schedule similar to what was used in the present study (Experiment 1). These joint findings suggest that responding for a reward-paired stimulus is elevated under PR conditions. Although food intake was restricted in the Chaudhri et. al., (2006a) study, a very mild restriction was used, and rats did not endure any loss of BW. Therefore, we are hesitant to consider their findings as further support for the lack of an effect of food restriction on performance under PR that is reinforced by reward-associated cues.

The lack of meaningful differences in responding under a PR schedule between sated and food-restricted rats can be potentially explained by the difficulty of the reinforcement schedule used. We remain unsure as to whether the reinforcement schedule was insufficiently or too challenging given that all rats quit responding before statistically significant differences developed between feeding groups. We exposed a subset of rats to a more challenging PR schedule (PR schedule = 2, 4, 7, 12, 17, 25, 36 ,50, 69, 95, 131, 178, 242, 328, 445, 603, 815, 1102, 1498, 2012…) derived from the formula: PR requirement = $[5e^{(\text{reward number X } 0.30)}]$-5 (Richardson & Roberts, 1996); not shown). No observable differences between feeding groups were demonstrated suggesting the schedule was too demanding. Furthermore, under a low effort schedule of reinforcement (FI 20 s), we identified a clear, food restriction effect on heroin seeking. Therefore, we suspect that the PR schedule used here was too challenging to be supported by cues only, which resulted in a lack of statistical difference between feeding conditions.

With respect to the sensitivity of the schedules, one methodological caveat that requires discussion pertains to *ratio strain*. Specifically, ratio strain, a disruption in behavioural responding resulting from a rapid elevation in response requirements, may have occurred given that rats were not gradually trained to respond on the PR schedule of reinforcement

(Aberman & Salamone, 1999). Ratio strain is well known to produce erratic behaviour, therefore the implementation of a training progression may be necessary in future investigations.

An alternative explanation may be that food restriction does not increase the conditioned rewarding properties of the heroin cues. Unfortunately, few studies have examined the role of PR on cues alone. However, we disagree with this alternative explanation given the strong effect caloric restriction on reward taking under PR schedules with BSR (Cabeza de Vaca, Krahne, & Carr, 2004), psychostimulant- (Carr, 2007; Li, He, Parrish, & Delich, 2003), and opiate-use (Li et al., 2003).

Typically, the ratio used in the present study has been a standard in the assessment of psychostimulant use, but not in terms of conditioned reinforcers. Therefore, the effect of food restriction on this ratio may be insensitive to the particular schedule employed here. Consequently, we assume that that our PR testing procedure was not ideal for the analysis of the motivational properties of drug cues in food-restricted rats, an issue that would require careful exploration in future studies.

An alternate approach to motivation: Investigating motivation through the acquisition of a novel operant response reinforced by heroin-associated cues (Experiment 2; Figure 2). In order to bypass the potential disadvantages of the PR schedule, we tested the acquisition of a novel operant behaviour reinforced by heroin cues. All rats showed preference for the heroin-cue-associated nose poke aperture compared to the alternative aperture, and food restriction augmented this preference compared to sated rats. These findings suggest that, in food-restricted rats, the augmentation in heroin seeking previously reported by our laboratory may in fact reflect an increase in the incentive motivational properties attributed to drug-associated cues (Sedki, Abbas, Angelis, Martin, et al., 2013a).

The present findings coincide with previous studies indicating that cues associated with cocaine can support the acquisition of a novel instrumental response across multiple tests (Di

Ciano et al., 2008; Di Ciano & Everitt, 2004; Samaha et al., 2011). In order to demonstrate that drug cues have acquired conditioned reinforcing properties, Di Ciano (2004) and Mackintosh (1974) argue that three criteria must be satisfied. First, conditioned, reward-seeking behaviour must be assessed independently of the operant measure associated with primary reinforcement. A large number of studies fail to satisfy this criterion given that the response paired with drug delivery is also used to measure drug seeking. Importantly, in both Di Ciano's (2004) research and in the present study (Experiment 2), the operant response performed during drug seeking was never one that was associated with drug delivery.

The second criterion aims to ensure that behaviour was not motivated by the location of the operant module, on the side of the chamber that was paired with drug delivery. This is unlikely in Di Ciano's (2004) and our procedure, given the relative locations of the active and alternative apertures were counterbalanced.

The final criterion asserts that the performance of operant responses result from the cue's association with the drug, and not from any inherent motivational properties of the cue. In order to accomplish this, Di Ciano and colleagues (2004) presented a cocaine-unpaired light stimulus during self-administration training. They reported that the cocaine-unpaired cue produced only weak reinforcement, which fulfills their criteria. However, the "weak" reinforcement described was equivalent to roughly 40% of the cocaine-paired cue responding, suggesting considerable intrinsic motivational properties. In addition, responding for a non-cocaine-paired cue was tested using a between-subjects design. As a consequence, any further analyses of choice behaviour, which may better elucidate the basis of responses, become impossible. This important caveat was addressed in experiments 3, 4, and 5.

The inconsistency observed between the acquisition of novel operant response data (Experiment 2) and behaviour under a PR schedule (Experiment 1) in the present thesis may reflect the distinct underlying mechanisms that support behaviour under these two paradigms.

While both procedures are believed to measure the underlying motivational properties of drug cues, they approach this common goal differently. For instance, a PR schedule of reinforcement measures the maximal effort a rat is willing to assign to a particular reward. Alternatively, the acquisition of a novel operant response considers the underlying conditioned rewarding properties of drug cues measured by their ability to support the learning of a novel behaviour. If the lack of any differences reported under a PR schedule are not dependent on the PR used, then perhaps food restriction does not affect the maximal effort to work for such cues as measured under this schedule. In contrast, food restriction may in fact facilitate the learning directed toward a novel operant response reinforced by the drug-associated cues. Investigations into the behavioural mechanisms that regulate food motivation lend further support for this idea, by emphasizing the distinction between effort-based and directional (approach)-based motivation (Salamone & Correa, 2009). For example, the effects of NAc DA depletion or antagonism (DA-D1- or -D2-like receptors) on food-reinforced behaviours have only been observed under high ratio requirements, with no observable disruptions in appetite or the motivation for primary food rewards (Kelley, Baldo, Pratt, & Will, 2005; Salamone, Kurth, McCullough, Sokolowski, & Cousins, 1993). Indeed, when food-restricted rats (~85% of free-feeding BW) were exposed to a concurrent choice task (preferred high-carbohydrate food pellets versus less-preferred standard laboratory chow)under a PR schedule, DA receptor antagonism reduced PR responding but not the general motivation for consumption (Randall et al., 2012). Interestingly, pre-feeding the rats reduced both PR responding and chow intake. In accordance with these findings, we have previously demonstrated that pre-feeding rats reduces the augmentation of heroin seeking (using a low ratio, FI-20 s schedule) in food-restricted rats, to levels observed in sated rats. This may suggest that a rat's motivational state is likely affected by food restriction (D'Cunha et al., 2013). Consequently, rats with NAc DA depletion exhibit typical approach and consummatory behaviour toward food, however showing reductions in the effort to work for food reward, and a bias toward obtaining food under low-effort tasks (Salamone & Correa, 2009). Based on these findings, we must consider the possibility that food restriction may not affect high ratio effort-based tasks such as the PR schedule of reinforcement. While this conflicts with data on the enhancing effect of food

restriction on PR tasks for BSR and opiate self-administration (Cabeza de Vaca et al., 2004; Li et al., 2003), it is important to note that these studies included a primary reward.

The role of food restriction in light-seeking behaviour (Experiment 3; Figure 3). Our first attempt to investigate the reinforcing effects of visual stimuli revealed modest overall increases in light seeking compared to responses on the alternative lever (Sedki, Abbas, Angelis, Martin, et al., 2013a). Furthermore, no feeding related changes were observed on lever responses. These data are inconsistent with early findings that responses for non-paired visual stimuli are elevated in food-restricted rats (Davis, 1958; Hurwitz & De, 1958; Keller et al., 2014; Segal, 1959; Smith & Donahoe, 1966; Stewart, 1960). It is difficult to consolidate these differences, however, as variations in methodology may explain the inconsistencies of these conclusions. For example, Keller et. al., (2014) demonstrated a food-restriction-induced potentiation of light seeking (primary reinforcement: light), but only after repeated testing. Our rats were tested during a single session. Therefore, it is possible that responding in food-restricted rats would have remained elevated, while responses performed by their sated counterparts would have declined steadily, following repeated exposure. In addition, we exposed animals to a gradual 14 day mild restriction resulting in a ~10% reduction in free-feeding BW. A similar food restriction protocol was used by Smith (1966) who aimed to restrict animals to 80% and 90% of their free-feeding BWs over a three-week period. While they reported a robust elevation in lever pressing under both restriction conditions compared to an unrestricted control group, the presentation of the light stimulus was unique to the final test session. In contrast, we trained our rats to respond for a light-stimulus reinforcement across 10 (3, 3 h/day) days of training. Therefore, Smith et al.'s (1966) findings may have been due to novelty rather than the inherent motivational properties of the light stimulus itself. Our data further suggest the improbability that the food-restriction-induced elevation in heroin seeking resulted from elevations in the motivational properties of the light cue per se. An important caveat is that current methodological designs do not directly compare food-restriction-induced changes in the motivational properties of heroin-conditioned cues versus alternative cues. The following experiment addressed this issue.

The acquisition of a novel behaviour driven by heroin, and non-heroin paired visual and auditory stimuli (Experiment 4; Figures 4-5). In a quest to verify the third criteria presented by Di Ciano et. al., (2004) we surprisingly discovered that the food-restriction-induced augmentation of heroin seeking (Experiment 2) was present, but weakened by the inherent motivational properties of the visual stimulus. The reduction of the food-restriction-induced augmentation of heroin seeking and the lack of a preference for the active aperture when the alternative nose poke was paired with a tone+light (Sated group, Experiment 4A), suggest that the incentive motivational properties of light cues may interact with drug-seeking behaviours. Furthermore, when the alternative stimulus consisted solely of a tone (Experiment 4B), both the food-restriction-induced augmentation of heroin seeking and the preference for the active aperture were re-established.

In order to understand the nature of these behavioural changes, several pertinent methodological limitations need to be considered. First, we cannot confirm that the attenuation in responding (Experiment 4A) was due to the light cue or the synergistic effects of the compound (light+tone) stimulus as a whole. In one study investigating the self-administration of visual and compound (auditory+visual) stimuli, it was found that mice respond more for complex compound stimuli (Olsen & Winder, 2012). Accordingly, See and colleagues (1999) also demonstrated that compound stimuli can maintain greater levels of reinstatement. However, they argue that the visual component alone may acquire greater incentive motivational properties outside the context of the compound cue. More recently, Shin (Shin, Cao, Webb, & Ikemoto, 2010) has corroborated this idea with data suggesting that visual, but not auditory, signals were able to facilitate seeking following non-contingent vehicle or amphetamine injections. While it remains difficult to draw comparisons across studies given the variation in both the stimulus parameters and behavioural procedures, we believe that the evidence supports our interpretation that the light stimulus is responsible for the attenuation of responses in Experiment 4A. However, a direct pairing of an alternative module with a light stimulus alone is required to confirm this.

Another procedural limitation includes the potential effect of overlap between the active and alternative cue presentation. As the alternative lever was non-retractable, drug and cue delivery following an active response may have immediately preceded the alternative response, at which time the alternative cues would activate in parallel. We contend that this did not disrupt learning, as rats underwent five days (3, 3 h sessions/day) of training, and the daily, total average of cue overlap across lever type was approximately 80 sec. It is unlikely that a contingency would develop following a brief exposure to the overlapped cues. This is especially true given that responses on the alternative lever occurred first in some cases.

Lastly, we did not directly measure the rats' ability to distinguish between cues, therefore future experiments would be necessary to resolve this limitation. However, we can propose several reasons to explain why cue generalizability may not be applicable to our findings. First, even if rats could not discriminate between the types of visual stimuli presented, the auditory cues were quite different (tone, white noise, and clicker). Second, the housing of the cues was distinct in shape, and therefore likely discernible. Lastly, the active and alternative modules in Experiment 4A were paired with a different combination of light/auditory cues. If rats could not discriminate between the visual cues, we believe they would likely be capable of differentiating between the combinations presented.

A matter of choice (Experiment 5; Figure 6). When presented with a choice task in which a commitment to a choice was not necessary (FR1+5 min ITI), all rats demonstrated a preference for the active compared to alternative lever. Furthermore, active lever responding was not affected by the feeding condition. We suggest that these null findings may be due to the lack of commitment in the schedule of reinforcement used. First, a ceiling effect may have limited our ability to observe any meaningful differences, as the maximum number of responses possible within a 3 h session is fixed. Furthermore, some responses may have resulted from impulsivity, and not from the commitment to the choice itself. Following a given response, lever accessibility was immediately forfeited, and erroneous responding could not be rectified. Indeed, compared to sated rats, augmented active lever responding was observed in food-

restricted rats following the implementation of a lean schedule of reinforcement (FR3+5 min ITI). The novelty of this schedule demanded greater commitment to the response, therefore may have reduced impulsivity toward the presented alternatives (Domjan, 2014). Collectively, these data suggest the necessity of using a strict choice procedure that allows for a committed choice in order to observe differences in drug and light seeking, as opposed to the simultaneous response availability.

Conclusion. The aforementioned experiments were designed to investigate the role of caloric restriction on the incentive motivational properties of heroin cues. Despite previous findings suggesting that the inherent motivational properties of light cues affect the degree to which caloric restrictions augment heroin seeking, our data indicate that food restriction may actually enhance the motivational properties of the drug-associated cues.

Chapter 5: GENERAL DISCUSSION

In this thesis, we have examined the underlying hormonal, neuronal, and behavioural mechanisms that mediate augmented heroin seeking in food-restricted rats. These perspectives were investigated for two important reasons. First, to understand the mechanisms of drug relapse using a newly developed measure (D'Cunha et al., 2013), which was designed to improve the translational validity of the commonly used reinstatement procedure. Second, we hoped that a multifocal approach would establish the groundwork to direct future research in our laboratory. To that end, three sets of experiments were included, each aimed to target the different processes that may modulate heroin seeking in calorically restricted rats.

Chapter 2. The first approach was motivated by the reported impact of drug use in women, and the potential influence of biological sex-differences (e.g., hormones) on abuse liability and treatment (Becker, McClellan, & Reed, 2017). Despite these indications, women are poorly represented in addiction research (Lynch, 2017), which has recently prompted several funding directives (National Institute of Health, Canadian Institute of Health and Research) to award more female-inclusive proposals. Due to the detrimental effects of drug abuse in women in combination with the lack of female representation in addiction research, we used female rats to assess the role of ovarian hormones in chronically food-restricted, heroin-seeking rats (Sedki, Gregory, Luminare, D'Cunha, & Shalev, 2015). We demonstrated that ovarian hormones contribute to, but are not necessary for, the augmentation of heroin seeking in food-restricted female rats. Though our consideration of female behaviour was exclusive to this chapter, we note the importance of further investigations to extend our findings in male rats to females as well.

Chapter 3. Here, our goal was to study an underlying target mechanism that may contribute to heroin-seeking behaviour in calorically restricted rats. Specifically, the glutamatergic system may be particularly relevant to the protracted withdrawal period in our procedure (Kalivas, 2009; Kalivas et al., 2009; Spencer, Scofield, & Kalivas, 2016). Previous research (D'Cunha et

al., 2017; Gipson & Kalivas, 2016; Spencer et al., 2016; Tobin, Sedki, Abbas, & Shalev, 2013) suggests that these changes may occur within the accumbal and pre-frontal cortex structures. As such, we measured the expression of the glutamate GluA1 receptor subunit in calorically restricted drug-naïve and heroin-trained male rats. We observed a reduction in GluA1 receptor expression in the NAc shell. To our surprise, we did not find any other differences in GluA1 receptor expression in the NAc core, IL or PL. We believe that this resulted from the methodological procedure used, and that techniques which evaluate the cell surface expression of the GluA1 receptor are required to confidently assess the receptors' role in calrodically restricted, heroin-seeking rats.

Chapter 4. The final approach was designed to validate our measurement. Therefore, male rats were used to investigate the motivational mechanisms that provoke, and maintain, heroin seeking. Specifically, we were interested in whether the reported heroin-cue seeking in food-restricted rats was a function of the cue's association with the drug, or whether it reflected an enhanced value of light cues that is independent of any drug association. Our findings indicated that caloric restriction contributes to the motivational properties attributed to light cues, due to their association with heroin.

Collectively, the data presented in this thesis demonstrate an invaluable approach to the understanding of novel methodology in translational addiction research. As is typical in any area of research, the answers to our initial hypotheses have developed into new questions (discussed below), which will guide future investigations in our laboratory.

5.1 - The hormonal approach: Understanding the role of ovarian hormones in food-restriction-induced drug-cue seeking

There is considerable evidence that addiction can differentially affect males and females (Becker et al., 2017; Becker & Koob, 2016; Carroll & Lynch, 2016; Sanchis Segura & Becker, 2016; Venniro et al., 2017). Indeed, compared to males, females escalate to addiction more

rapidly, and are more susceptible to relapse (Becker & Hu, 2008; Becker & Koob, 2016; Lynch et al., 2002). It has been proposed that variations in circulating ovarian hormones contribute to this enhanced vulnerability to drugs of abuse (Becker & Koob, 2016). Pre-clinically, the limited number of studies conducted on the role of ovarian hormones in heroin-trained rats has indicated that E2 may facilitate, while progesterone may limit, drug intake (Lacy et al., 2016). To our knowledge, we are the first (Sedki et al., 2015; Chapter 2) to address the role of ovarian hormones in heroin seeking using food-restricted female rats.

5.1.1 - Methodological considerations

To begin, we present two limitations to our methodology that warrant discussion. The first involves the impact of natural hormone fluctuations during heroin training. In our procedure, blood samples were exclusively acquired on test-day. As such, we could not assess the impact of estrous cycle fluctuations on drug intake and "experience" during consumption. However, we can speculate that the temporal duration of our training procedure allowed rats to experience each phase of the estrous cycle, yet we do not know whether heroin administration disrupted the cycle itself. Previous research in rodents (Mavrikaki, Pravetoni, Page, Potter, & Chartoff, 2017), but not humans (Santen, Sofsky, Bilic, & Lippert, 1975), indicates that no disruptions in the ovarian cycle were observed upon exposure to opiate use. Therefore, we contend that disruptions in our rats' ovarian cycle resulting from heroin administration is unlikely. In our effort to control hormone fluctuation, we also note that we ignored both the importance of hormonal levels and the time-dependent shift in such levels. It has been suggested that these natural changes in hormonal levels are especially relevant when evaluating the effects of sex hormones on behaviour (Fuchs et al., 2005). Though we do not report any observable disruptions in the estrous cycle, we wish to highlight its importance when investigating heroin-taking and -seeking behaviours.

Second, we attempted to control hormonal fluctuations by OVX. However, we did not consider the influence of other organs on the circulating levels of sex hormones. For example, the

adrenal glands act as another source of progesterone (Fajer, Holzbauer, & Newport, 1971), and E2 can promote the synthesis of neural progesterone (Micevych, Soma, & Sinchak, 2008). Therefore, we cannot ignore the presence of circulating progesterone in our ovariectomized, E2-treated rats. However, we believe it is unlikely that this impacted our results, as supra-physiological doses of progesterone did not produce any significant changes in behaviour (Chapter 2).

5.1.2 - Estradiol the suppressor: The role of anxiety and hunger in the attenuation of food-restriction-induced heroin seeking in estradiol-treated rats.

In Chapter 2, we argued that the attenuation of heroin seeking in chronically food-restricted, E2-treated rats, did not result from E2's anxiolytic influence (Sedki et al., 2015). This stemmed from our finding that the food-restriction-induced augmentation in heroin seeking observed was not due to an increased physiological stress response in male rats (Sedki, Abbas, Angelis, Martin, et al., 2013a). However, the aforementioned evaluated an acute (test day) blockade of the stress response, and not the prolonged impact of stress experienced during withdrawal. Furthermore, these data do not account for the sex differences in stress vulnerability that warrant further discussion here. For example, rats with high levels of E2 show decreased anxiety-like behaviours compared to rats with lower E2 levels and males (Frye & Walf, 2002; Frye, Petralia, & Rhodes, 2000; Mora, Dussaubat, & Díaz-Véliz, 1996). We have not directly evaluated the stress response in female rats, therefore we cannot dismiss the differential role of the stress response in our male and female rats. However, we note that heroin seeking was only reduced in the food-restricted, E2-treated group. If stress provoked heroin seeking, then we would expect increased heroin seeking in the sated/ovariectomized/cholesterol-treated, compared to the E2-treated rats. While further studies are needed to justify these conclusions, we contend that the attenuation of food-restriction-induced heroin seeking in E2-treated rats is better clarified through anorectic mechanisms (Asarian & Geary, 2006; 2013; Butera, 2010). For example, E2 has a tonic inhibitory effect on food intake throughout the estrous cycle, where higher levels of E2 are followed by decreases in food intake (Asarian & Geary, 2006). In

addition, E2 has been shown to reverse (Asarian & Geary, 2002; Butera, Wojcik, & Clough, 2010) the increase in both BW and food intake following OVX (Wade & Gray, 1979). Therefore, it is likely that the effect of E2 on augmented heroin seeking in food-restricted rats is due to its actions on feeding-related mechanisms.

5.1.3 - The potential role of estradiol receptors in disruption of food-restriction-induced heroin seeking.

The effects of E2 are mediated by its binding to one of two estrogen receptors: ERα and ERβ, the former of which is believed to regulate E2's effects on energy balance and feeding. For instance, ERα (Lemieux et al., 2005), but not ERβ (Ohlsson et al., 2000) -knockout mice have been shown to weigh 30% more, and have a 50% greater body fat composition compared to wild type mice. One explanation for this discrepancy, is that the ERα may mediate feeding behaviour through satiety. For example, chronic treatment with an ERα, but not ERβ agonist, prevents both the overeating and weight gain that is typically observed in ovariectomized rats (Roesch, 2006). Furthermore, ERα's weight management effects appear to be specific to portion control, rather than meal frequency (Santollo, Wiley, & Eckel, 2007). This becomes particularly relevant in our research given that satiation, through acute re-feeding, reverses the effects of chronic food restriction on heroin seeking in male rats (D'Cunha et al., 2013). ERα's effects on satiety are also reflected by the motivation to work for food rewards. For example, ERα-knockout mice are less likely to work for food at higher prices (increased fixed-ratio response requirement) compared to wild type mice (Minervini, Rowland, Robertson, & Foster, 2015). Therefore, it is possible that activation of ERα in our food-restricted, E2-treated rats attenuated the hunger state, which in turn reduced the motivation for heroin seeking.

5.1.4 - Sex and hunger: The interaction between estradiol and the "hunger" hormones.

In chronically food-restricted male rats, intra-VTA treatment with JMV 2959 (an antagonist to

the orexigenic gut hormone, ghrelin), dose-dependently decreases heroin seeking (D'Cunha, Russo, Le Noble, Rizzo, Daoud, et al., & Shalev, 2018). These data coincide with the idea that hunger state is a critical factor in heroin seeking in chronically food-restricted male rats. In females, the satiety may partially depend on interactions between estrogen and endogenous gut hormones. Though ovariectomized rats exhibit increased feeding following intra-ventricular/peripheral administration of ghrelin (Nakazato, Murakami, Date, & Kojima, 2001), these effects are blocked by treatment with E2 (Clegg et al., 2007). In accordance, estrogen replacement has also been shown to decrease the elevations of ghrelin mRNA, which are observed following OVX (Matsubara et al., 2004). In naturally cycling rats, treatment with ghrelin only increases feeding during diestrus (low E2), and not the proestrus or estrous (high E2) phases of the estrous cycle (Clegg et al., 2007). In mice lacking the growth hormone secretagogue receptor (GHSR), increases in food intake and BW were not observed following OVX (Clegg et al., 2007). Therefore, it remains possible that the removal of ovaries releases the tonic inhibition of ghrelin by E2, which in turn stimulates hunger and promotes weight gain. While no studies have directly investigated the effects of ERα on ghrelin, it has been shown that ERα is co-localized on ghrelin-immunopositive cells (Matsubara et al., 2004). Therefore, E2 may directly impact the regulation of ghrelin in female rats. These actions may be regulated by ERα and GHSRs, however, future studies need to identify the exact nature of this interaction. Collectively, these data suggest that E2 may have suppressed heroin seeking in our food-restricted groups by weakening the orexigenic effects of ghrelin.

Leptin, secreted by the adipose cells, is known to tonically inhibit eating and increase energy expenditure (Ahima, Kelly, Elmquist, & Flier, 1999; Friedman, 2009; Myers, Münzberg, Leinninger, & Leshan, 2009). In ovariectomized rats, E2 treatment increases the eating-inhibitory effects of leptin (Ainslie et al., 2001; Clegg, Riedy, Smith, Benoit, & Woods, 2003). In support, intra-ventricular infusions of leptin attenuate feeding in intact, but not ovariectomized rats (Clegg, Brown, Woods, & Benoit, 2006). Thus, E2 may have a direct effect on leptin sensitivity in female rats. In our ovariectomized rats, E2 attenuated food-restriction-induced

heroin seeking, suggesting that E2 enhanced the anorexigenic effects of leptin to reduce satiety and limit heroin seeking.

Another possibility is that E2 may act by upregulating the leptin receptor-b (LRb), which occurs in E2-treated rats (Rocha, Bing, Williams, & Puerta, 2004). The specific mechanism by which E2 promotes the upregulation of the LRb, however, remains unknown. One aspect worth considering involves the role of the ERα, which is co-localized with the LRb (Diano, Kalra, Sakamoto, & Horvath, 1998). However, the physiological relevance of these effects and changes are unclear, as leptin does not vary according to the estrus cycle in naturally cycling rats (Eckel et al., 1998).

5.1.5 - Conclusions

Estradiol may not be necessary for the food-restriction-induced augmentation of heroin seeking, however, its role in attenuating this behaviour provides an alternative approach to treatment in females. While we have addressed certain questions pertaining to how sex hormones affect hunger and heroin seeking, the underlying mechanisms of this effect remain unclear. Specifically, the way estrogen receptors and gut hormones interact to promote and inhibit heroin seeking in chronically food-restricted rats, are unknown.

5.2 - The neuronal approach: Understanding the role of glutamate receptors in food-restriction-induced drug-cue seeking

The GluA2-lacking AMPARs within the NAc and mPFC are critical to drug-induced plasticity (Boudreau & Wolf, 2005; Kourrich et al., 2007; Mammen et al., 1997; Shen & Kalivas, 2013), cue-induced heroin seeking (Tobin & Shalev, 2012), and caloric restriction (Carr, 2016; Ouyang et al., 2017). Despite these indications, we failed to identify a meaningful role for the GluA1-R in the food-restriction-induced augmentation of heroin seeking (Chapter 3). Below, we discuss

alternate glutamatergic targets and the involvement of the GluA1-R in the glutamate system, in calorically restricted heroin-seeking rats.

5.2.1 The "wrong" receptor: The potential role for alternative glutamate receptors.

Reports on the expression of the transcription factor ΔFosB contradict the assertion that GluA2-lacking AMPARs are the primary glutamatergic subunits involved in the modulation of drug-seeking behaviours. Compared to wild-types, mice over-expressing ΔFosB in the NAc exhibit behaviours which are comparable to chronically drug-exposed mice. Specifically, these "ΔFosB" mice express a willingness to work for lower doses of cocaine (Colby, Whisler, Steffen, Nestler, & Self, 2003) and display an overall reduction of the reward threshold in both cocaine- (Kelz et al., 1999) and morphine-conditioned (Zachariou et al., 2006) environments. Therefore, an over expression of ΔFosB may cause mice to become more sensitive to the incentive motivational properties of abused drugs. Evidence using non-transgenic animals also supports this notion, as an accumulation of ΔFosB is observed in response to a variety of abused drugs (e.g., cocaine, ethanol, morphine) in the NAc core and shell, and the mPFC (Nestler, 2008; Perrotti et al., 2008). In fact, Nestler (2008) has previously suggested that the long-term stability of the ΔFosB isoform allows ΔFosB to function as a persistent molecular switch that maintains drug addiction following prolonged withdrawal. Along with the induction of ΔFosB, a concomitant rise in GluR2-R expression is also observed (Kelz et al., 1999), indicating that a rise in GluA2-lacking AMPARs is somehow accompanied by an elevation in GluA2-R expression. Though recent efforts have begun to dissect this contradiction in cocaine exposed rats (Grueter, Robison, & Neve, 2013), no studies have addressed this in heroin-seeking animals. This provides an interesting direction for subsequent studies on the role of GluA2 in heroin seeking, which we have previously suggested to be secondary to the functioning of the GluA2-lacking AMPARs (Chapter 3).

Little is known on the effects of mGluRs in opiate-reward-related behaviours. The few studies that have evaluated mGluRs in opiate-related behaviours have reported distinct findings dependent on the particular receptor targeted. For example, treatment with MPEP and MTEP (i.e., negative allosteric modulators [NAMs] of the mGluR5) demonstrates that the indirect blockade of mGluR5 attenuates cue-induced heroin seeking (Brown, Stagnitti, Duncan, & Lawrence, 2012; Lou, Chen, Liu, Ruan, & Zhou, 2014) and heroin self-administration (Brown et al., 2012) in rodents. Interestingly, MTEP has also been shown to reduce food intake in rats (Bradbury et al., 2005), therefore it is possible that the administration of an mGluR5 antagonist may completely eliminate drug seeking in our food-restricted rats. However, we advise caution as both MPEP and MTEP are known to produce significant off-target effects (Lea & Faden, 2006). Nonetheless, the role of mGluR5s must be re-evaluated using target-specific allosteric modulators. Alternatively, treatment with an mGluR2/3 agonist (Bossert, Poles, Sheffler-Collins, & Ghitza, 2006b) and treatment with an mGluR7 positive allosteric modulator (Li, Xi, & Markou, 2013) were shown to reduce heroin self-administration and heroin-seeking behaviour. In addition, systemic (Bossert, Busch, & Gray, 2005) and intra-NAc injections (Bossert, Gray, Lu, & Shaham, 2006a) of the mGluR2/3 agonist LY379268 also attenuated the reinstatement of cue-induced heroin seeking. These data are particularly interesting given the role of mGluR2/3 in reducing glutamate release (Schoepp, 2001), which in turn, is elevated upon re-exposure to heroin cues (Wang, You, & Wise, 2012). However, it remains unclear how mGlur2, mGluR3, and mGluR7s are involved in feeding-related behaviours, though it appears that both activating mGluR2, mGluR3, and mGluR7 and mGluR5 blockade act to reduce heroin-seeking behaviour. To that end, if agonists to the mGluR2, mGluR3, and mGluR7 promotes feeding, then their administration in our rats may be counterintuitive. Therefore, we emphasize the relevance of future studies in determining their role in feeding behaviour.

5.2.2 In defence of the GluA1 receptor, from a dopaminergic perspective.

The study of mGluRs present an interesting approach for our laboratory. We also contend that AMPARs remain highly relevant to our procedure, despite the null findings observed in our food-restricted rats. Previously, we expressed the importance of DA-D1-Rs in the augmentation of heroin seeking in food-restricted rats under withdrawal, where antagonism of DA-D1-like receptors was found to attenuate this behaviour (D'Cunha et al., 2017). Interestingly, one of the downstream effects of DA-D1-R activation includes the trafficking, phosphorylation, and insertion of AMPARs into the cell membrane surface (Barry & Ziff, 2002; Gao & Wolf, 2007). Indeed, the administration of a DA-D1-like receptor agonist into the NAc was found to increase AMPAR insertion into extra-synaptic regions (Sun, Milovanovic, Zhao, & Wolf, 2008). In support, the cocaine-induced facilitation of glutamate release in the VTA has been associated with an upregulation of DA-D1-R signaling, which can be blocked by DA-D1-like receptor antagonism (Kalivas & Duffy, 1998). Importantly, the effect of DA-D1-Rs in the food-restriction-induced augmentation of heroin seeking (Glovaci et al., 2018) indirectly support a significant role for AMPARs.

In addition to the finding that NAc core DA-D1-like receptor antagonism can inhibit the augmentation of heroin seeking in food-restricted rats, we have also reported elevations in extracellular levels of DA in the NAc core in heroin-seeking, food-restricted rats (D'Cunha et al., 2017). Furthermore, DA release within the NAc often results from elevated VTA transmission. Recently, research using optogenetics has demonstrated that these VTA DA-projections to the NAc core and shell co-release glutamate and DA, an effect believed to be mediated by AMPARs (Stuber, Hnasko, Britt, Edwards, & Bonci, 2010). Based on the strong evidence presented above (Chapter 3) and the involvement of the dopaminergic system in our food-restricted rats, we therefore contend that the role of AMPARs requires further evaluation. Importantly, we suggest that the null findings we have demonstrated result from methodological rather than conceptual issues.

5.2.4 - Conclusions

In our attempt to address how the GluA1-R affects the augmentation of heroin seeking by food restriction, we have introduced many new questions pertaining to the actions of the glutamatergic system. Though unlikely, we consider the possibility that the GluA1 receptor is not involved in the expression of heroin seeking in chronically food-restricted rats, and suggest other (e.g., GluA2-R, mGluRs) potential targets for study. We are deeply excited at the prospect of using alternative methods to address the limitations of our approach. The evaluation of cell surface expression could be the first steps in deciphering the precise involvement of the GluA1-R in chronically food-restricted, heroin-seeking rats.

5.3 - The behavioural approach: Understanding the modulation of cue value in the food-restriction-induced augmentation of drug-cue seeking

Drug-cue reactivity is an area of research that has been widely studied in addiction and is largely derived from Pavlovian conditioning. For instance, neutral stimuli paired with the delivery of a drug reward (unconditioned stimuli; US) become associated with the effects of the drug, or come to predict the availability of the drug (conditioned stimuli; CS). In absence of the drug these CS elicit conditioned responses (CR), which are similar to those previously reserved for the drug reward itself (unconditioned responses; UR). Here (Chapter 4), our particular interest involves the strong motivational state observed in the presence of reward-associated cues, which can then act as incentive stimuli to facilitate reward seeking (Robinson, Yager, Cogan, & Saunders, 2014; Stewart, de Wit, & Eikelboom, 1984). Specifically, we seek to understand how the motivational elements attributed to incentive stimuli are changed in calorically-restricted states.

In Chapter 4, we demonstrated that dietary restrictions enhance the motivational properties attributed to heroin-associated cues. However, our approach was incomplete in addressing the fundamental properties that define incentive stimuli and the individual variation observed in our heroin-seeking rats, both of which are discussed below.

5.3.1 The missing elements: Does food restriction enhance the incentive salience attributed to heroin cues?

Incentive stimuli have been broadly defined by the existing literature. First, by their relative attractiveness or ability to elicit cue-targeted approaches. Second, in terms of their capacity to act as conditioned reinforcers to facilitate, maintain, or enhance reward seeking, and lastly, by their ability to trigger conditioned motivation in the absence of a primary reward (see Figure 5.1; Milton & Everitt, 2010; Robinson et al., 2014; Valyear, Villaruel, & Chaudhri, 2017). In heroin-trained rats, cues can reliably trigger drug seeking (Shaham et al., 2003), however, our understanding of how incentive value is modified in heroin-trained rats or during calorically-restricted states is poorly understood. In fact, the incentive value of opiate cues has been largely understudied (Madsen & Ahmed, 2015; Peters & De Vries, 2014; Yager, Pitchers, Flagel, & Robinson, 2015), and few reports have addressed the added influence of caloric restriction.

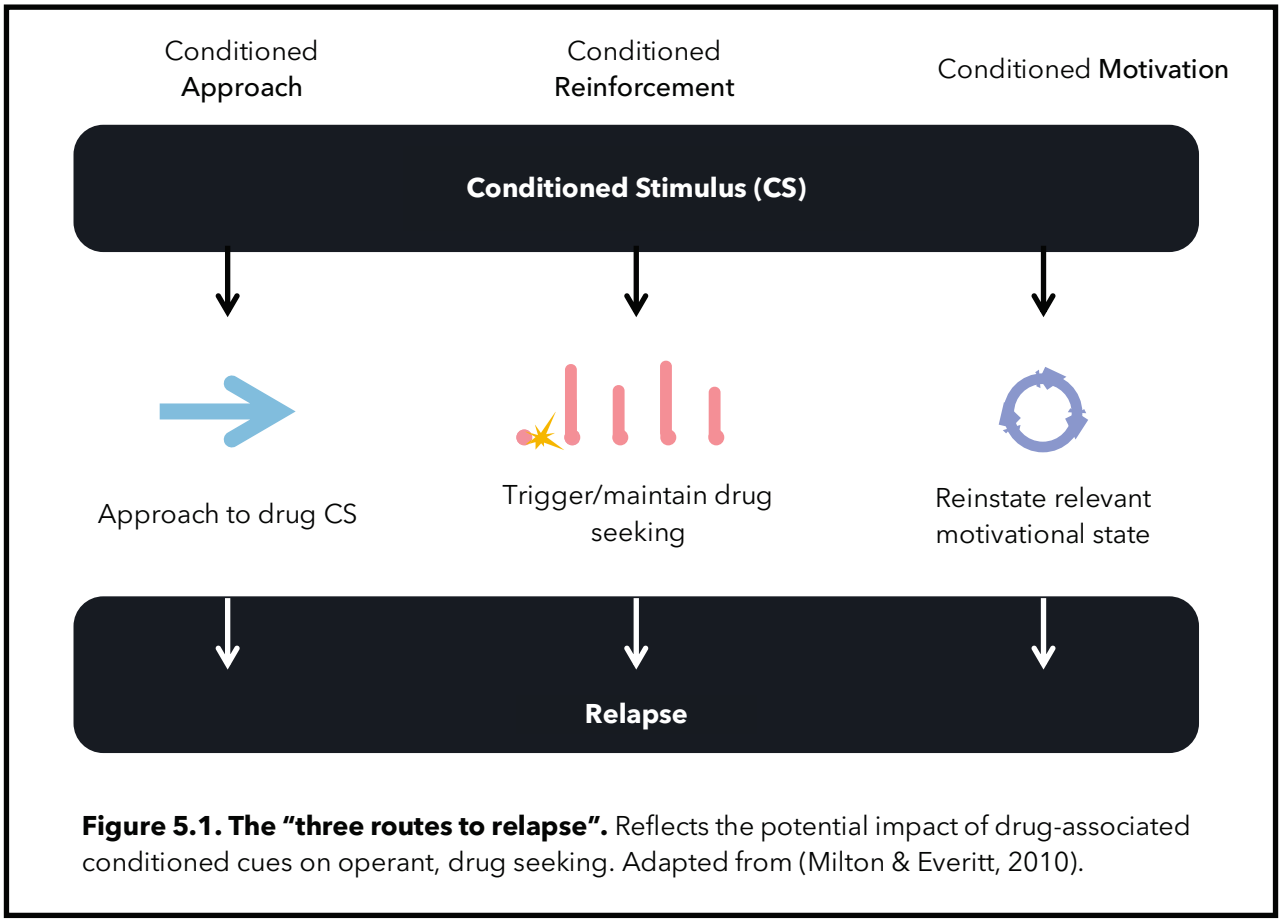

Figure 5.1. The "three routes to relapse". Reflects the potential impact of drug-associated conditioned cues on operant, drug seeking. Adapted from (Milton & Everitt, 2010).

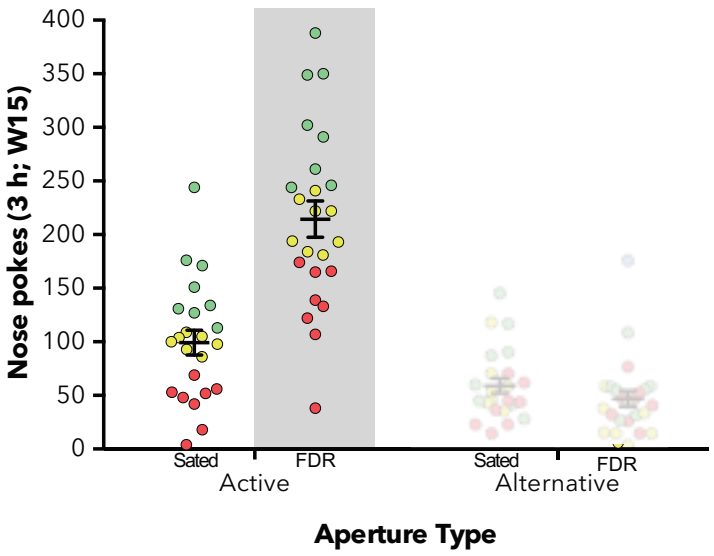

Figure 5.2. Individual Variation, a return to Figure 4.2D. The above figure is a recreation of Figure 4.2D, reflecting the number of nose poke responses made on W15, in the active and alternative (faded) apertures. Green dots represent the top 33%, Yellow dots represent the middle 33%, while red dots represent the bottom 33% number of nose pokes committed by the Sated and food-restricted (FDR) rats.

Conditioned approach behaviours have been observed in the context of intravenously-administered drugs, including those directed toward psychostimulant (Uslaner, Acerbo, Jones, & Robinson, 2006; Yager & Robinson, 2013) and opiate cues (Madsen & Ahmed, 2015; Peters & De Vries, 2014; Yager et al., 2015). In terms of conditioned-approach behaviours and opiate cues, two important considerations must be addressed before we attempt to extend these findings to our research (Madsen & Ahmed, 2015; Peters & De Vries, 2014; Yager et al., 2015). First, the long-lasting physiological effects of heroin may hinder CS-US learning in the standard multi-trial conditioning approach. To resolve this issue, we must either consider a single, one-day conditioning session (Peters & De Vries, 2014) which would extend the duration of conditioning, or the use of short-lasting opiates (Yager et al., 2015). Second, a higher dose of heroin (or a more potent opiate) may be needed to produce similar neurophysiological effects following non-contingent treatment as those observed during the self-administration procedure (Lee, Criado, Koob, & Henriksen, 1999). In the aforementioned studies, the ability for heroin cues to trigger conditioned approach behaviour suggests that they have become more attractive due to a relative increase in their incentive value. However, the role of caloric restriction in modulating conditioned approach behaviour toward such heroin cues remains unclear. Though we observed rigorous interaction with the both the cue-light and lever in our

rats, we have not gathered quantifiable data to support this explanation. As such, we remain cautious in our interpretation. We also wish to highlight that future investigations are necessary to clarify whether we have satisfied the first criteria with regards to the ability of heroin cues to elicit conditioned approach behaviours.

Drug cues can also act as conditioned reinforcers. Furthermore, they have been shown to trigger drug seeking in animal models of relapse, and to contribute to the persistence of drug-seeking behaviour following long periods of withdrawal (Venniro et al., 2016). An interesting and invaluable demonstration pertaining to drug cues acting as conditioned reinforcers is shown in the acquisition of a novel instrumental response for the presentation of the drug cue alone (Di Ciano et al., 2008; Di Ciano & Everitt, 2004; Palmatier et al., 2007; Samaha et al., 2011). We have demonstrated that heroin cues can act as conditioned reinforcers, in that they both trigger and maintain drug seeking (D'Cunha et al., 2017; Sedki, Abbas, Angelis, Martin, et al., 2013a). Furthermore, heroin cues also support the acquisition of a novel instrumental response targeted at the drug cue alone (Chapter 4). Importantly, our data suggest that chronic food restriction enhances the incentive value attributed to incentive stimuli. As such, we believe that this satisfies the second characteristic of conditioned reinforcement.

Incentive stimuli are also defined by their ability to prompt conditioned motivational states in the absence of the primary reward. These motivational states are believed to initiate relapse, and to contribute to the maintenance and augmentation of drug seeking and taking. The Pavlovian-instrumental transfer (PIT) procedure is commonly used to measure the motivational state of a conditioned stimulus (CS) through its influence on instrumental behaviour (Balleine, 1994; Hall, Parkinson, Connor, Dickinson, & Everitt, 2001; Rescorla & Solomon, 1967). Pavlovian-instrumental transfer involves three phases. In phase one, a cue and reinforcer are paired. Next, an instrumental response is performed to earn the same reinforcer that was paired in the first phase. Lastly, instrumental responses are measured under extinction conditions, in the presence of the conditioned cue. It is expected that an increase in instrumental responses would be observed. There is considerable evidence for the motivational influence of natural

rewards, (Crombag, Galarce, & Holland, 2008b; Holmes, Marchand, & Coutureau, 2010; Rescorla, 1994) and a few studies have examined psychostimulant drug rewards using PIT (LeBlanc, Maidment, & Ostlund, 2014; LeBlanc, Ostlund, & Maidment, 2012). To our knowledge, PIT has not been used to investigate heroin cues. While some reports restricted feeding to enhance the training for food rewards (Holmes et al., 2010), no studies have directly measured the effects of food restriction on PIT. Future studies need to adopt the PIT procedure to elucidate whether heroin cues can instigate conditioned motivational states, and whether this state is influenced by food restriction.

5.3.2 Individual Variation: Are all food-restricted rats created equal?

Heroin cues trigger seeking behaviours through their ability to act as incentive stimuli (Di Ciano & Everitt, 2004). However, individual variations exist in the degree to which drug cues both acquire incentive motivation properties and act as incentive stimuli to drive drug seeking. Based on our findings, we observed individual differences in responding for the heroin cue, however we are unable to interpret the source of this variability. Instead, we can speculate on the nature of these individual differences and propose future studies to clarify these differences (**Figure 5.2**).

Once a CS has been paired with a reward, rodents can be categorized according to their behavioural approach. For instance, certain rats display conditioned approach behaviour toward the CS (i.e., sign-trackers). In others, the CS may trigger approach toward the goal itself (i.e., reward-delivery site; goal-trackers). Lastly, a subset of rats exhibit no preference in conditioned approach behaviour (Flagel, Akil, & Robinson, 2009). In one study designed to assess individual differences in the propensity for a cue to acquire incentive salience, augmented food seeking was observed in sign-trackers, compared to goal-trackers (Saunders & Robinson, 2010). Interestingly, sign-trackers also displayed a heightened incentive salience for cocaine cues when compared to goal-trackers (Saunders & Robinson, 2010). Given the absence of a reward-delivery site, our focus on intravenously-administered heroin makes it

difficult to identify goal-trackers. However, a solution to rectify this issue has recently been proposed. Specifically, a conditioned orienting response, equally observed in sign- and goal-tracking rats, can be differentiated from a conditioned approach response, which is observed to a greater degree in sign-tracking rats (Saunders & Robinson, 2012; Yager et al., 2015; Yager & Robinson, 2013). Our laboratory emphasizes these important considerations for future studies.

To date, only one study has examined individual differences in behavioural approach upon the presentation of opiate cues. Similar to evidence reported with food and psychostimulant cues, opiate cues also carry higher incentive value in sign-trackers, compared to goal-trackers (Yager et al., 2015). Therefore, we expect that rats in our study that are categorized as sign-trackers may also attribute greater value to the heroin cues. However, it remains unclear whether food restriction increases heroin seeking in sign-trackers to a proportionally greater extent than it may in goal-trackers. For example, it is possible that food-restricted goal-trackers may behave similarly to sign-trackers with free access to chow. Though these suggestions have yet to be clarified, they provide an interesting direction for future studies in our laboratory.

5.3.3 A comment on unintentional findings: Should we turn off the lights?

Our findings suggest that the conditioned rewarding properties of drug cues are strongly influenced by the inherent motivational properties of the cue lights they are associated with. In fact, competing visual stimuli are especially disruptive under simple schedules of reinforcement and choice procedures, where responses require low commitment. Rats allowed unrestricted access to chow appear to be more susceptible to the inherent motivational properties of visual stimuli, resulting in identical responding for a drug-paired and non-drug-paired light; some small differences in responding remain apparent in food-restricted rats. Therefore, we argue that using visual stimuli as reward-associated stimuli should be approached cautiously. It should also be noted that we do not advocate for their abandonment but emphasize their consideration in research given two very important points. First, proper control conditions need

to be implemented to establish that observable changes in reward seeking are not due to the effects of visual stimuli. Second, manipulations (i.e., food restriction) need to be verified to ensure that they do not directly alter responding to visual stimuli, apart from their target reward associations. These points are critical given the observed differences in responding resulting from the specific procedure and schedule, discussed previously. Importantly, we suggest that schedules requiring minimal effort should be reconsidered when visual stimuli are employed. Lastly, investigations into whether the use of auditory stimuli may be sufficient to measure drug-seeking behaviour could also bypass the methodological issues resulting from the inclusion of visual stimuli.

5.4 Conclusion

The current thesis used multiple approaches to understand the role of dietary restriction in heroin seeking. Each set of experiments was uniquely designed to expand on a particular perspective concerning three fundamental avenues of behavioural neuroscience research; hormonal, neuronal, and behavioural mechanisms. While our findings have answered several questions on the role of ovarian sex hormones, the glutamatergic system, and the behavioural properties that underlie the augmentation of heroin seeking in chronically food-restricted rats, we have inspired many questions that will be the focus of future research in our laboratory.

REFERENCES

Aberman, J. E., & Salamone, J. D. (1999). Nucleus accumbens dopamine depletions make rats more sensitive to high ratio requirements but do not impair primary food reinforcement. *Nsc*, *92*(2), 545–552. http://doi.org/10.1016/S0306-4522(99)00004-4

Aghajanian, G. K., Kogan, J. H., & Moghaddam, B. (1994). Opiate withdrawal increases glutamate and aspartate efflux in the locus coeruleus: an in vivo microdialysis study. *Brain Research*, *636*(1), 126–130.

Ahima, R. S., Kelly, J., Elmquist, J. K., & Flier, J. S. (1999). Distinct Physiologic and Neuronal Responses to Decreased Leptin and Mild Hyperleptinemia 1. *Endocrinology*, *140*(11), 4923–4931. http://doi.org/10.1210/endo.140.11.7105

Ainslie, D. A., Morris, M. J., Wittert, G., Turnbull, H., Proietto, J., & Thorburn, A. W. (2001). Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. *International Journal of Obesity (2005)*, *25*(11), 1680–1688. http://doi.org/10.1038/sj.ijo.0801806

Aitken, T. J., Greenfield, V. Y., & Wassum, K. M. (2016). Nucleus accumbens core dopamine signaling tracks the need-based motivational value of food-paired cues. *Journal of Neurochemistry*, *136*(5), 1026–1036. http://doi.org/10.1111/jnc.13494

Allen, A. M., Lunos, S., Heishman, S. J., al'Absi, M., Hatsukami, D., & Allen, S. S. (2015). Subjective response to nicotine by menstrual phase. *Addictive Behaviors*, *43*, 50–53. http://doi.org/10.1016/j.addbeh.2014.12.008

Alleweireldt, A. T., Weber, S. M., Kirschner, K. F., Bullock, B. L., & Neisewander, J. L. (2002). Blockade or stimulation of D1 dopamine receptors attenuates cue reinstatement of extinguished cocaine-seeking behavior in rats. *Psychopharmacology*, *159*(3), 284–293. http://doi.org/10.1007/s002130100904

Almey, A., Hafez, N. M., Hantson, A., & Brake, W. G. (2013). Deficits in latent inhibition induced by estradiol replacement are ameliorated by haloperidol treatment. *Frontiers in Behavioral Neuroscience*, *7*, 136. http://doi.org/10.3389/fnbeh.2013.00136

Anker, J. J., & Carroll, M. E. (2010). Sex differences in the effects of allopregnanolone on yohimbine-induced reinstatement of cocaine seeking in rats. *Drug and Alcohol Dependence*, *107*(2-3), 264–267. http://doi.org/10.1016/j.drugalcdep.2009.11.002

Anker, J. J., Holtz, N. A., Zlebnik, N., & Carroll, M. E. (2009). Effects of allopregnanolone on the reinstatement of cocaine-seeking behavior in male and female rats. *Psychopharmacology*, *203*(1), 63–72. http://doi.org/10.1007/s00213-008-1371-9

Anker, J. J., Larson, E. B., Gliddon, L. A., & Carroll, M. E. (2007). Effects of progesterone on the reinstatement of cocaine-seeking behavior in female rats. *Experimental and Clinical Psychopharmacology*, *15*(5), 472–480. http://doi.org/10.1037/1064-1297.15.5.472

Arnold, J. M., & Roberts, D. C. (1997). A critique of fixed and progressive ratio schedules used to examine the neural substrates of drug reinforcement. *Pharmacology, Biochemistry and Behavior*, *57*(3), 441–447.

Asarian, L., & Geary, N. (2002). Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. *Hormones and Behavior*, *42*(4), 461–471.

Asarian, L., & Geary, N. (2006). Modulation of appetite by gonadal steroid hormones. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*(1471), 1251–1263. http://doi.org/10.1098/rstb.2006.1860

Asarian, L., & Geary, N. (2013). Sex differences in the physiology of eating. *AJP: Regulatory, Integrative and Comparative Physiology*, *305*(11), R1215–67. http://doi.org/10.1152/ajpregu.00446.2012

Association, A. P. (2013). Diagnostic and Statistical Manual of Mental Disorders (DSM-5®). American Psychiatric Pub.

Balleine, B. (1994). Asymmetrical interactions between thirst and hunger in Pavlovian-instrumental transfer. *The Quarterly Journal of Experimental Psychology. B, Comparative and Physiological Psychology*, *47*(2), 211–231.

Barry, M. F., & Ziff, E. B. (2002). Receptor trafficking and the plasticity of excitatory synapses. *Current Opinion in Neurobiology*, *12*(3), 279–286. http://doi.org/10.1016/S0959-4388(02)00329-X

Bäckström, P., & Hyytiä, P. (2006). Ionotropic and metabotropic glutamate receptor antagonism attenuates cue-induced cocaine seeking. *Neuropsychopharmacology*, *31*(4), 778–786. http://doi.org/10.1038/sj.npp.1300845

Becker, J. B., & Hu, M. (2008). Sex differences in drug abuse. *Frontiers in Neuroendocrinology*, *29*(1), 36–47. http://doi.org/10.1016/j.yfrne.2007.07.003

Becker, J. B., & Koob, G. F. (2016). Sex Differences in Animal Models: Focus on Addiction. *Pharmacological Reviews*, *68*(2), 242–263. http://doi.org/10.1124/pr.115.011163

Becker, J. B., McClellan, M. L., & Reed, B. G. (2017). Sex differences, gender and addiction. *Journal of Neuroscience Research*, *95*(1-2), 136–147. http://doi.org/10.1002/jnr.23963

Becker, J. B., Perry, A. N., & Westenbroek, C. (2012). Sex differences in the neural mechanisms mediating addiction: a new synthesis and hypothesis. *Biology of Sex Differences*, *3*(1), 14. http://doi.org/10.1186/2042-6410-3-14

Bell, S. M., Stewart, R. B., Thompson, S. C., & Meisch, R. A. (1997). Food-deprivation increases cocaine-induced conditioned place preference and locomotor activity in rats. *Psychopharmacology*, *131*(1), 1–8.

Berridge, K. C., Robinson, T. E., & Aldridge, J. W. (2009). Dissecting components of reward: 'liking', 'wanting', and learning. *Current Opinion in Pharmacology*, *9*(1), 65–73. http://doi.org/10.1016/j.coph.2008.12.014

Blumenthal, D. M., & Gold, M. S. (2010). Neurobiology of food addiction. *Current Opinion in Clinical Nutrition & Metabolic Care*, *13*(4), 359–365. http://doi.org/10.1097/MCO.0b013e32833ad4d4

Bossert, J. M., Busch, R. F., & Gray, S. M. (2005). The novel mGluR2/3 agonist LY379268 attenuates cue-induced reinstatement of heroin seeking. *Neuroreport*, *16*(9), 1013–1016.

Bossert, J. M., Gray, S. M., Lu, L., & Shaham, Y. (2006a). Activation of group II metabotropic glutamate receptors in the nucleus accumbens shell attenuates context-induced relapse to heroin seeking. *Neuropsychopharmacology*, *31*(10), 2197–2209. http://doi.org/10.1038/sj.npp.1300977

Bossert, J. M., Poles, G. C., Sheffler-Collins, S. I., & Ghitza, U. E. (2006b). The mGluR2/3 agonist LY379268 attenuates context- and discrete cue-induced reinstatement of sucrose seeking

but not sucrose self-administration in rats. *Behavioural Brain Research*, *173*(1), 148–152. http://doi.org/10.1016/j.bbr.2006.06.008

Bossert, J. M., Poles, G. C., Wihbey, K. A., Koya, E., & Shaham, Y. (2007). Differential Effects of Blockade of Dopamine D1-Family Receptors in Nucleus Accumbens Core or Shell on Reinstatement of Heroin Seeking Induced by Contextual and Discrete Cues. *Journal of Neuroscience*, *27*(46), 12655–12663. http://doi.org/10.1523/JNEUROSCI.3926-07.2007

Boudreau, A. C., & Wolf, M. E. (2005). Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *25*(40), 9144–9151. http://doi.org/10.1523/JNEUROSCI.2252-05.2005

Bowers, M. S., Chen, B. T., & Bonci, A. (2010). AMPA Receptor Synaptic Plasticity Induced by Psychostimulants: The Past, Present, and Therapeutic Future. *Neuron*, *67*(1), 11–24. http://doi.org/10.1016/j.neuron.2010.06.004

Bowman, R. E., Ferguson, D., & Luine, V. N. (2002). Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. *Nsc*, *113*(2), 401–410. http://doi.org/10.1016/S0306-4522(02)00156-2

Bradbury, M. J., Campbell, U., Giracello, D., Chapman, D., King, C., Tehrani, L., et al. (2005). Metabotropic Glutamate Receptor mGlu5 Is a Mediator of Appetite and Energy Balance in Rats and Mice. *The Journal of Pharmacology and Experimental Therapeutics*, *313*(1), 395–402. http://doi.org/10.1124/jpet.104.076406

Bradley, M. M., Cuthbert, B. N., & Lang, P. J. (1993). Pictures as prepulse: attention and emotion in startle modification. *Psychophysiology*, *30*(5), 541–545. http://doi.org/10.1111/j.1469-8986.1993.tb02079.x

Brady, K. T., & Randall, C. L. (1999). Gender differences in substance use disorders. *The Psychiatric Clinics of North America*, *22*(2), 241–252.

Brown, R. M., Stagnitti, M. R., Duncan, J. R., & Lawrence, A. J. (2012). The mGlu5 receptor antagonist MTEP attenuates opiate self-administration and cue-induced opiate-seeking

behaviour in mice. *Drug and Alcohol Dependence*, *123*(1-3), 264–268. http://doi.org/10.1016/j.drugalcdep.2011.11.002

Butcher, R. L., Collins, W. E., & Fugo, N. W. (1974). Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17beta throughout the 4-day estrous cycle of the rat. *Endocrinology*, *94*(6), 1704–1708. http://doi.org/10.1210/endo-94-6-1704

Butera, P. C. (2010). Estradiol and the control of food intake. *Physiology & Behavior*, *99*(2), 175–180. http://doi.org/10.1016/j.physbeh.2009.06.010

Butera, P. C., Wojcik, D. M., & Clough, S. J. (2010). Effects of estradiol on food intake and meal patterns for diets that differ in flavor and fat content. *Physiology & Behavior*, *99*(1), 142–145. http://doi.org/10.1016/j.physbeh.2009.10.009

Cabeza de Vaca, S., & Carr, K. D. (1998). Food restriction enhances the central rewarding effect of abused drugs. *Journal of Neuroscience*, *18*(18), 7502–7510.

Cabeza de Vaca, S., Krahne, L. L., & Carr, K. D. (2004). A progressive ratio schedule of self-stimulation testing in rats reveals profound augmentation of d-amphetamine reward by food restriction but no effect of a "sensitizing" regimen of d-amphetamine. *Psychopharmacology*, *175*(1), 106–113. http://doi.org/10.1007/s00213-003-1768-4

Cabib, S., Orsini, C., Le Moal, M., & Piazza, P. V. (2000). Abolition and reversal of strain differences in behavioral responses to drugs of abuse after a brief experience. *Science*, *289*(5478), 463–465.

Caggiula, A. R., Donny, E. C., Chaudhri, N., Perkins, K. A., Evans-Martin, F. F., & Sved, A. F. (2002). Importance of nonpharmacological factors in nicotine self-administration. *Physiology & Behavior*, *77*(4-5), 683–687.

Caggiula, A. R., Donny, E. C., White, A. R., Chaudhri, N., Booth, S., Gharib, M. A., et al. (2001). Cue dependency of nicotine self-administration and smoking. *Pharmacology, Biochemistry and Behavior*, *70*(4), 515–530. http://doi.org/10.1016/S0091-3057(01)00676-1

Campbell, U. C., & Carroll, M. E. (2001). Effects of ketoconazole on the acquisition of intravenous cocaine self-administration under different feeding conditions in rats. *Psychopharmacology*, *154*(3), 311–318. http://doi.org/10.1007/s002130000627

Carr, K. D. (2002). Augmentation of drug reward by chronic food restriction: behavioral evidence and underlying mechanisms. *Physiology & Behavior*, *76*(3), 353–364.

Carr, K. D. (2007). Chronic food restriction: enhancing effects on drug reward and striatal cell signaling. *Physiology & Behavior*, *91*(5), 459–472. http://doi.org/10.1016/j.physbeh.2006.09.021

Carr, K. D. (2016). Nucleus accumbens AMPA receptor trafficking upregulated by food restriction: an unintended target for drugs of abuse and forbidden foods. *Current Opinion in Behavioral Sciences*, *9*, 32–39. http://doi.org/10.1016/j.cobeha.2015.11.019

Carroll, M. E. (1985). The role of food deprivation in the maintenance and reinstatement of cocaine-seeking behavior in rats. *Drug and Alcohol Dependence*, *16*(2), 95–109.

Carroll, M. E., & Anker, J. J. (2010). Sex differences and ovarian hormones in animal models of drug dependence. *Hormones and Behavior*, *58*(1), 44–56. http://doi.org/10.1016/j.yhbeh.2009.10.001

Carroll, M. E., & Lynch, W. J. (2016). How to study sex differences in addiction using animal models. *Addiction Biology*, *21*(5), 1007–1029. http://doi.org/10.1111/adb.12400

Carroll, M. E., & Meisch, R. A. (1981). Determinants of increased drug self-administration due to food deprivation. *Psychopharmacology*, *74*(3), 197–200.

Carroll, M. E., & Meisch, R. (1984). Increased drug-reinforced behavior due to food deprivation. *Behavioral Brain Research*, *4*. http://doi.org/10.1016/B978-0-12-004704-8.50008-0

Carroll, M. E., Campbell, U. C., & Heideman, P. (2001). Ketoconazole suppresses food restriction-induced increases in heroin self-administration in rats: Sex differences. *Experimental and Clinical Psychopharmacology*, *9*(3), 307–316. http://doi.org/10.1037//1064-1297.9.3.307

Carter, B. L., & Tiffany, S. T. (1999). Meta-analysis of cue-reactivity in addiction research. *Addiction*, *94*(3), 327–340.

Chaudhri, N., Caggiula, A. R., Donny, E. C., Booth, S., Gharib, M., Craven, L., et al. (2006a). Operant responding for conditioned and unconditioned reinforcers in rats is differentially enhanced by the primary reinforcing and reinforcement-enhancing effects of nicotine. *Psychopharmacology*, *189*(1), 27–36. http://doi.org/10.1007/s00213-006-0522-0

Chaudhri, N., Caggiula, A. R., Donny, E. C., Palmatier, M. I., Liu, X., & Sved, A. F. (2006b). Complex interactions between nicotine and nonpharmacological stimuli reveal multiple roles for nicotine in reinforcement. *Psychopharmacology*, *184*(3-4), 353–366. http://doi.org/10.1007/s00213-005-0178-1

Chen, B. T., Hopf, F. W., & Bonci, A. (2010). Synaptic plasticity in the mesolimbic system: therapeutic implications for substance abuse. *Annals of the New York Academy of Sciences*, *1187*(1), 129–139. http://doi.org/10.1111/j.1749-6632.2009.05154.x

Chen, W., Wang, Y., Sun, A., Zhou, L., Xu, W., Zhu, H., et al. (2016). Activation of AMPA receptor in the infralimbic cortex facilitates extinction and attenuates the heroin-seeking behavior in rats. *Neuroscience Letters*, *612*, 126–131. http://doi.org/10.1016/j.neulet.2015.11.024

Cheskin, L. J., Hess, J. M., Henningfield, J., & Gorelick, D. A. (2005). Calorie restriction increases cigarette use in adult smokers. *Psychopharmacology*, *179*(2), 430–436. http://doi.org/10.1007/s00213-004-2037-x

Childress, A. R., Hole, A. V., Ehrman, R. N., Robbins, S. J., McLellan, A. T., & O'Brien, C. P. (1993). Cue reactivity and cue reactivity interventions in drug dependence. *NIDA Research Monograph*, *137*, 73–95.

Childs, E., & de Wit, H. (2009). Amphetamine-induced place preference in humans. *Biological Psychiatry*, *65*(10), 900–904. http://doi.org/10.1016/j.biopsych.2008.11.016

Childs, E., & de Wit, H. (2013). Contextual conditioning enhances the psychostimulant and incentive properties of d-amphetamine in humans. *Addiction Biology*, *18*(6), 985–992. http://doi.org/10.1111/j.1369-1600.2011.00416.x

Cicero, T. J., Aylward, S. C., & Meyer, E. R. (2003). Gender differences in the intravenous self-administration of μ opiate agonists. *Pharmacology, Biochemistry and Behavior*, *74*(3), 541–9. http://doi.org/10.1016/S0091-3057(02)01039-0

Clegg, D. J., Brown, L. M., Woods, S. C., & Benoit, S. C. (2006). Gonadal Hormones Determine Sensitivity to Central Leptin and Insulin. *Diabetes*, *55*(4), 978–987. http://doi.org/10.2337/diabetes.55.04.06.db05-1339

Clegg, D. J., Brown, L. M., Zigman, J. M., Kemp, C. J., Strader, A. D., Benoit, S. C., et al. (2007). Estradiol-Dependent Decrease in the Orexigenic Potency of Ghrelin in Female Rats. *Diabetes*, *56*(4), 1051–1058. http://doi.org/10.2337/db06-0015

Clegg, D. J., Riedy, C. A., Smith, K. A. B., Benoit, S. C., & Woods, S. C. (2003). Differential Sensitivity to Central Leptin and Insulin in Male and Female Rats. *Diabetes*, *52*(3), 682–687. http://doi.org/10.2337/diabetes.52.3.682

Colby, C. R., Whisler, K., Steffen, C., Nestler, E. J., & Self, D. W. (2003). Striatal Cell Type-Specific Overexpression of ΔFosB Enhances Incentive for Cocaine. *Journal of Neuroscience*, *23*(6), 2488–2493. http://doi.org/10.1126/science.282.5387.298

Collingridge, G. L., Isaac, J. T. R., & Wang, Y. T. (2004). Receptor trafficking and synaptic plasticity. *Nature Reviews Neuroscience*, *5*(12), 952–962. http://doi.org/10.1038/nrn1556

Conason, A., Teixeira, J., Hsu, C.-H., Puma, L., Knafo, D., & Geliebter, A. (2013). Substance use following bariatric weight loss surgery. *JAMA Surgery*, *148*(2), 145–150. http://doi.org/10.1001/2013.jamasurg.265

Conrad, K. L., Tseng, K. Y., Uejima, J. L., Reimers, J. M., Heng, L.-J., Shaham, Y., et al. (2008). Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. *Nature*, *454*(7200), 118–121. http://doi.org/10.1038/nature06995

Cooper, Z. D., & Haney, M. (2014). Investigation of sex-dependent effects of cannabis in daily cannabis smokers. *Drug and Alcohol Dependence*, *136*, 85–91. http://doi.org/10.1016/j.drugalcdep.2013.12.013

Corbit, L. H., Janak, P. H., & Balleine, B. W. (2007). General and outcome-specific forms of Pavlovian-instrumental transfer: the effect of shifts in motivational state and inactivation of the ventral tegmental area. *European Journal of Neuroscience*, *26*(11), 3141–3149. http://doi.org/10.1111/j.1460-9568.2007.05934.x

Cornish, J. L., & Kalivas, P. W. (2000). Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. *Journal of Neuroscience*, *20*(15), RC89.

Crombag, H. S., Bossert, J. M., Koya, E., & Shaham, Y. (2008a). Context-induced relapse to drug seeking: a review. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1507), 3233–3243. http://doi.org/10.1098/rstb.2008.0090

Crombag, H. S., Galarce, E. M., & Holland, P. C. (2008b). Pavlovian influences on goal-directed behavior in mice: the role of cue-reinforcer relations. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *15*(5), 299–303. http://doi.org/10.1101/lm.762508

Culbert, K. M., Racine, S. E., & Klump, K. L. (2016). Hormonal Factors and Disturbances in Eating Disorders. *Current Psychiatry Reports*, *18*(7), 65. http://doi.org/10.1007/s11920-016-0701-6

Cummings, J. A., Gowl, B. A., Westenbroek, C., Clinton, S. M., Akil, H., & Becker, J. B. (2011). Effects of a selectively bred novelty-seeking phenotype on the motivation to take cocaine in male and female rats. *Biology of Sex Differences*, *2*(1), 3. http://doi.org/10.1186/2042-6410-2-3

D'Cunha, T. M., Daoud, E., Rizzo, D., Bishop, A. B., Russo, M., Mourra, G., et al. (2017). Augmentation of Heroin Seeking Following Chronic Food Restriction in the Rat: Differential Role for Dopamine Transmission in the Nucleus Accumbens Shell and Core. *Neuropsychopharmacology*, *42*(5), 1136–1145. http://doi.org/10.1038/npp.2016.250

D'Cunha, T., Russo, M., Le Noble, S., Rizzo, D., Daoud, E., Sedki, F., & Shalev, U. (2018). A role for leptin and ghrelin in the augmentation of heroin seeking induced by chronic food restriction. *Unpublished*.

D'Cunha, T. M., Sedki, F., Macri, J., Casola, C., & Shalev, U. (2013). The effects of chronic food restriction on cue-induced heroin seeking in abstinent male rats. *Psychopharmacology*, *225*(1), 241–250. http://doi.org/10.1007/s00213-012-2810-1

D'Souza, M. S. (2015). Glutamatergic transmission in drug reward: implications for drug addiction. *Frontiers in Neuroscience*, *9*, 404. http://doi.org/10.3389/fnins.2015.00404

Dallman, M. F., Akana, S. F., Bhatnagar, S., Bell, M. E., Choi, S., Chu, A., et al. (1999). Starvation: early signals, sensors, and sequelae. *Endocrinology*, *140*(9), 4015–4023. http://doi.org/10.1210/endo.140.9.7001

Davis, J. D. (1958). The reinforcing effect of weak-light onset as a function of amount of food deprivation. *Journal of Comparative and Physiological Psychology*. http://doi.org/10.1037/h0049158

De Jaeger, X., Bishop, S. F., Ahmad, T., Lyons, D., Ng, G. A., & Laviolette, S. R. (2013). The effects of AMPA receptor blockade in the prelimbic cortex on systemic and ventral tegmental area opiate reward sensitivity. *Psychopharmacology*, *225*(3), 687–695. http://doi.org/10.1007/s00213-012-2852-4

de Wit, H., & Stewart, J. (1981a). Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology*, *75*(2), 134–143. http://doi.org/10.1007/BF00432175

de Wit, H., & Stewart, J. (1981b). Reinstatement of cocaine-reinforced responding in the rat. *Psychopharmacology*, *75*(2), 134–143.

Derkach, V. A., Oh, M. C., Guire, E. S., & Soderling, T. R. (2007). Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nature Reviews Neuroscience*, *8*(2), 101–113. http://doi.org/10.1038/nrn2055

Deroche-Gamonet, V. (2004). Evidence for Addiction-like Behavior in the Rat. *Science*, *305*(5686), 1014–1017. http://doi.org/10.1126/science.1099020

Deroche-Gamonet, V., Piat, F., Le Moal, M., & Piazza, P. V. (2002). Influence of cue-conditioning on acquisition, maintenance and relapse of cocaine intravenous self-administration. *The European Journal of Neuroscience*, *15*(8), 1363–1370.

Di Ciano, P. (2008). Distinct contributions of dopamine receptors in the nucleus accumbens core or shell to established cocaine reinforcement under a second-order schedule. *European Neuropsychopharmacology*, *18*(12), 888–896. http://doi.org/10.1016/j.euroneuro.2008.07.007

Di Ciano, P., & Everitt, B. J. (2004). Conditioned reinforcing properties of stimuli paired with self-administered cocaine, heroin or sucrose: implications for the persistence of addictive behaviour. *Neuropharmacology*, *47 Suppl 1*, 202–213. http://doi.org/10.1016/j.neuropharm.2004.06.005

Di Ciano, P., Robbins, T. W., & Everitt, B. J. (2008). Differential effects of nucleus accumbens core, shell, or dorsal striatal inactivations on the persistence, reacquisition, or reinstatement of responding for a drug-paired conditioned reinforcer. *Neuropsychopharmacology*, *33*(6), 1413–1425. http://doi.org/10.1038/sj.npp.1301522

Diano, S., Kalra, S. P., Sakamoto, H., & Horvath, T. L. (1998). Leptin receptors in estrogen receptor-containing neurons of the female rat hypothalamus. *Brain Research*, *812*(1-2), 256–259. http://doi.org/10.1016/S0006-8993(98)00936-6

Dingledine, R., Borges, K., Bowie, D., & Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacological Reviews*. http://doi.org/10.1002/(ISSN)2052-1707

Domjan, M. (2014). The Principles of Learning and Behavior. Nelson Education.

Donny, E. C., Caggiula, A. R., Rowell, P. P., Gharib, M. A., Maldovan, V., Booth, S., et al. (2000). Nicotine self-administration in rats: estrous cycle effects, sex differences and nicotinic receptor binding. *Psychopharmacology*, *151*(4), 392–405.

Eckel, L. A., Langhans, W., Kahler, A., Campfield, L. A., Smith, F. J., & Geary, N. (1998). Chronic administration of OB protein decreases food intake by selectively reducing meal size in female rats. *The American Journal of Physiology*, *275*(1 Pt 2), R186–93.

Edler, C., Lipson, S. F., & Keel, P. K. (2007). Ovarian hormones and binge eating in bulimia nervosa. *Psychological Medicine*, *37*(1), 131–141. http://doi.org/10.1017/S0033291706008956

Epstein, D. H., Preston, K. L., Stewart, J., & Shaham, Y. (2006). Toward a model of drug relapse: an assessment of the validity of the reinstatement procedure. *Psychopharmacology*, *189*(1), 1–16. http://doi.org/10.1007/s00213-006-0529-6

Epstein, D. H., Willner-Reid, J., Vahabzadeh, M., Mezghanni, M., Lin, J.-L., & Preston, K. L. (2009). Real-Time Electronic Diary Reports of Cue Exposure and Mood in the Hours Before Cocaine and Heroin Craving and Use. *Archives of General Psychiatry*, *66*(1), 88–94. http://doi.org/10.1001/archgenpsychiatry.2008.509

Evans, S. M., & Foltin, R. W. (2010). Does the response to cocaine differ as a function of sex or hormonal status in human and non-human primates? *Hormones and Behavior*, *58*(1), 13–21. http://doi.org/10.1016/j.yhbeh.2009.08.010

Evans, S. M., Haney, M., & Foltin, R. W. (2002). The effects of smoked cocaine during the follicular and luteal phases of the menstrual cycle in women. *Psychopharmacology*, *159*(4), 397–406. http://doi.org/10.1007/s00213-001-0944-7

Everitt, B. J., & Robbins, T. W. (2016). Drug Addiction: Updating Actions to Habits to Compulsions Ten Years On. *Annual Review of Psychology*, *67*, 23–50. http://doi.org/10.1146/annurev-psych-122414-033457

Fajer, A. B., Holzbauer, M., & Newport, H. M. (1971). The contribution of the adrenal gland to the total amount of progesterone produced in the female rat. *The Journal of Physiology*, *214*(1), 115–126. http://doi.org/10.1113/jphysiol.1971.sp009422

Fatseas, M., Denis, C., Massida, Z., Verger, M., Franques-Rénéric, P., & Auriacombe, M. (2011). Cue-induced reactivity, cortisol response and substance use outcome in treated heroin dependent individuals. *Biological Psychiatry*, *70*(8), 720–727. http://doi.org/10.1016/j.biopsych.2011.05.015

Fatseas, M., Serre, F., Alexandre, J. M., Debrabant, R., Auriacombe, M., & Swendsen, J. (2015). Craving and substance use among patients with alcohol, tobacco, cannabis or heroin addiction: a comparison of substance- and person-specific cues. *Addiction*, *110*(6), 1035–1042. http://doi.org/10.1111/add.12882

Feltenstein, M. W., Byrd, E. A., Henderson, A. R., & See, R. E. (2009). Attenuation of cocaine-seeking by progesterone treatment in female rats. *Psychoneuroendocrinology*, *34*(3), 343–352. http://doi.org/10.1016/j.psyneuen.2008.09.014

Feltenstein, M. W., Henderson, A. R., & See, R. E. (2011). Enhancement of cue-induced reinstatement of cocaine-seeking in rats by yohimbine: sex differences and the role of the estrous cycle. *Psychopharmacology*, *216*(1), 53–62. http://doi.org/10.1007/s00213-011-2187-6

Flagel, S. B., Akil, H., & Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology*, *56*, 139–148. http://doi.org/10.1016/j.neuropharm.2008.06.027

Franklin, J. C., Schiele, B. C., Brozek, J., & Keys, A. (1948). Observations on human behavior in experimental semistarvation and rehabilitation. *Journal of Clinical Psychology*, *4*(1), 28–45. http://doi.org/10.1002/1097-4679(194801)4:1<28::AID-JCLP2270040103>3.0.CO;2-F

Franklin, T. R., Jagannathan, K., Wetherill, R. R., Johnson, B., Kelly, S., Langguth, J., et al. (2015). Influence of menstrual cycle phase on neural and craving responses to appetitive smoking

cues in naturally cycling females. *Nicotine & Tobacco Research : Official Journal of the Society for Research on Nicotine and Tobacco*, *17*(4), 390–397. http://doi.org/10.1093/ntr/ntu183

Friedman, J. M. (2009). Leptin at 14 y of age: an ongoing story. *The American Journal of Clinical Nutrition*, *89*(3), 973S–979S. http://doi.org/10.3945/ajcn.2008.26788B

Frye, C. A., & Walf, A. A. (2002). Changes in Progesterone Metabolites in the Hippocampus Can Modulate Open Field and Forced Swim Test Behavior of Proestrous Rats. *Hormones and Behavior*, *41*(3), 306–315. http://doi.org/10.1006/hbeh.2002.1763

Frye, C. A., Petralia, S. M., & Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α,5α-THP. *Pharmacology, Biochemistry and Behavior*, *67*(3), 587–596. http://doi.org/10.1016/S0091-3057(00)00392-0

Fuchs, R. A., Branham, R. K., & See, R. E. (2006). Different neural substrates mediate cocaine seeking after abstinence versus extinction training: a critical role for the dorsolateral caudate-putamen. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *26*(13), 3584–3588. http://doi.org/10.1523/JNEUROSCI.5146-05.2006

Fuchs, R. A., Evans, K. A., Mehta, R. H., Case, J. M., & See, R. E. (2005). Influence of sex and estrous cyclicity on conditioned cue-induced reinstatement of cocaine-seeking behavior in rats. *Psychopharmacology*, *179*(3), 662–672. http://doi.org/10.1007/s00213-004-2080-7

Fuchs, R. A., Lasseter, H. C., Ramirez, D. R., & Xie, X. (2008). Relapse to drug seeking following prolonged abstinence: the role of environmental stimuli. *Drug Discovery Today: Disease Models*, *5*(4), 251–258. http://doi.org/10.1016/j.ddmod.2009.03.001

Fulton, S., Woodside, B., & Shizgal, P. (2000). Modulation of brain reward circuitry by leptin. *Science*. http://doi.org/10.1126/science.287.5450.125

Gallop, R. J., Crits-Christoph, P., Have, Ten, T. R., Barber, J. P., Frank, A., Griffin, M. L., & Thase, M. E. (2007). Differential transitions between cocaine use and abstinence for men and women. *Journal of Consulting and Clinical Psychology*, *75*(1), 95–103. http://doi.org/10.1037/0022-006X.75.1.95

Gao, C., & Wolf, M. E. (2007). Dopamine alters AMPA receptor synaptic expression and subunit composition in dopamine neurons of the ventral tegmental area cultured with prefrontal cortex neurons. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *27*(52), 14275–14285. http://doi.org/10.1523/JNEUROSCI.2925-07.2007

Gass, J. T., & Olive, M. F. (2008). Glutamatergic substrates of drug addiction and alcoholism. *Biochemical Pharmacology*, *75*(1), 218–265. http://doi.org/10.1016/j.bcp.2007.06.039

Gawin, F. H., & Kleber, H. D. (1986). Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. *Archives of General Psychiatry*, *43*(2), 107–113.

Geier, A., Mucha, R. F., & Pauli, P. (2000). Appetitive nature of drug cues confirmed with physiological measures in a model using pictures of smoking. *Psychopharmacology*, *150*(3), 283–291. http://doi.org/10.1007/s002130000404

Geiger, B. M., Haburcak, M., Avena, N. M., Moyer, M. C., Hoebel, B. G., & Pothos, E. N. (2009). Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity. *Neuroscience*, *159*(4), 1193–1199. http://doi.org/10.1016/j.neuroscience.2009.02.007

Gipson, C. D., & Kalivas, P. W. (2016). Neural Basis of Drug Addiction. In *Drug Abuse in Adolescence* (pp. 37–56). Cham: Springer International Publishing. http://doi.org/10.1007/978-3-319-17795-3_4

Gipson, C. D., Kupchik, Y. M., & Kalivas, P. W. (2014). Rapid, transient synaptic plasticity in addiction. *Neuropharmacology*, *76 Pt B*, 276–286. http://doi.org/10.1016/ j.neuropharm.2013.04.032

Gipson, C. D., Kupchik, Y. M., Shen, H., Reissner, K. J., Thomas, C. A., & Kalivas, P. W. (2013). Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. *Neuron*, *77*(5), 867–872. http://doi.org/10.1016/j.neuron.2013.01.005

Glass, M. J., Lane, D. A., Colago, E. E. O., Chan, J., Schlussman, S. D., Zhou, Y., et al. (2008). Chronic administration of morphine is associated with a decrease in surface AMPA GluR1 receptor subunit in dopamine D1 receptor expressing neurons in the shell and non-D1 receptor expressing neurons in the core of the rat nucleus accumbens. *Experimental Neurology*, *210*(2), 750–761. http://doi.org/10.1016/j.expneurol.2008.01.012

Glovaci, I., D'Cunha, T., Chapman, A., & Shalev, U. (2018). Enhanced NMDA receptor mediated responses in the nucleus accumbens shell following withdrawal from heroin self-administration is blocked by chronic food restriction. *Unpublished Manuscript*.

Goletiani, N. V., Siegel, A. J., Lukas, S. E., & Hudson, J. I. (2015). The effects of smoked nicotine on measures of subjective states and hypothalamic-pituitary-adrenal axis hormones in women during the follicular and luteal phases of the menstrual cycle. *Journal of Addiction Medicine*, *9*(3), 195–203. http://doi.org/10.1097/ADM.0000000000000117

Greenfield, S. F., Brooks, A. J., Gordon, S. M., Green, C. A., Kropp, F., McHugh, R. K., et al. (2007). Substance abuse treatment entry, retention, and outcome in women: a review of the literature. *Drug and Alcohol Dependence*, *86*(1), 1–21. http://doi.org/10.1016/j.drugalcdep.2006.05.012

Griffin, M. L., Weiss, R. D., Mirin, S. M., & Lange, U. (1989). A comparison of male and female cocaine abusers. *Archives of General Psychiatry*, *46*(2), 122–126.

Grimm, J. W., Hope, B. T., Wise, R. A., & Shaham, Y. (2001). Neuroadaptation. Incubation of cocaine craving after withdrawal. *Nature*, *412*(6843), 141–142. http://doi.org/10.1038/35084134

Grueter, B. A., Robison, A. J., & Neve, R. L. (2013). Δ FosB differentially modulates nucleus accumbens direct and indirect pathway function. Presented at the Proceedings of the …. http://doi.org/10.1073/pnas.1221742110/-/DCSupplemental

Gutman, A. L., Ewald, V. A., Cosme, C. V., Worth, W. R., & LaLumiere, R. T. (2016). The infralimbic and prelimbic cortices contribute to the inhibitory control of cocaine-seeking behavior during a discriminative stimulus task in rats. *Addiction Biology*. http://doi.org/10.1111/adb.12434

Hall, J., Parkinson, J. A., Connor, T. M., Dickinson, A., & Everitt, B. J. (2001). Involvement of the central nucleus of the amygdala and nucleus accumbens core in mediating Pavlovian influences on instrumental behaviour. *The European Journal of Neuroscience*, *13*(10), 1984–1992. http://doi.org/10.1046/j.0953-816x.2001.01577.x

Hall, S. M., Tunstall, C. D., Vila, K. L., & Duffy, J. (1992). Weight gain prevention and smoking cessation: cautionary findings. *American Journal of Public Health*, *82*(6), 799–803. Retrieved from /pmc/articles/PMC1694191/?report=abstract

Hanna, J. M., & Hornick, C. A. (1977). Use of coca leaf in southern Peru: Adaptation or addiction. *Bulletin on Narcotics*.

Harris, J. A., Jones, M. L., Bailey, G. K., & Westbrook, R. F. (2000). Contextual control over conditioned responding in an extinction paradigm. *Journal of Experimental Psychology. Animal Behavior Processes*, *26*(2), 174–185. http://doi.org/10.1037/0097-7403.26.2.174

Hasin, D. S., Stinson, F. S., Ogburn, E., & Grant, B. F. (2007). Prevalence, Correlates, Disability, and Comorbidity of DSM-IV Alcohol Abuse and Dependence in the United States: Results From the National Epidemiologic Survey on Alcohol and Related Conditions. *Archives of General Psychiatry*, *64*(7), 830–842. http://doi.org/10.1001/archpsyc.64.7.830

Hearing, M. C., Jedynak, J., Ebner, S. R., Ingebretson, A., Asp, A. J., Fischer, R. A., et al. (2016). Reversal of morphine-induced cell-type-specific synaptic plasticity in the nucleus accumbens shell blocks reinstatement. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(3), 757–762. http://doi.org/10.1073/pnas.1519248113

Hernandez-Avila, C. A., Rounsaville, B. J., & Kranzler, H. R. (2004). Opioid-, cannabis- and alcohol-dependent women show more rapid progression to substance abuse treatment. *Drug and Alcohol Dependence*, *74*(3), 265–272. http://doi.org/10.1016/j.drugalcdep.2004.02.001

Hodos, W. (1961). Progressive Ratio as a Measure of Reward Strength. *Science*, *134*(3483), 943–944. http://doi.org/10.1126/science.134.3483.943

Hogarth, L., Balleine, B. W., Corbit, L. H., & Killcross, S. (2013). Associative learning mechanisms underpinning the transition from recreational drug use to addiction. *Annals of the New York Academy of Sciences*, *1282*(1), 12–24. http://doi.org/10.1111/j.1749-6632.2012.06768.x

Holderness, C. C., & Brooks-Gunn, J. (1994). Eating disorders and substance use: a dancing vs a nondancing population. *Medicine & Science in* …. http://doi.org/10.1249/00005768-199403000-00005

Hollmann, M., Hartley, M., & Heinemann, S. (1991). Ca2+ permeability of KA-AMPA--gated glutamate receptor channels depends on subunit composition. *Science*, *252*(5007), 851–853.

Holmes, N. M., Marchand, A. R., & Coutureau, E. (2010). Pavlovian to instrumental transfer: a neurobehavioural perspective. *Neuroscience and Biobehavioral Reviews*, *34*(8), 1277–1295. http://doi.org/10.1016/j.neubiorev.2010.03.007

Hser, Y. I., Anglin, M. D., & Booth, M. W. (1987). Sex differences in addict careers. 3. Addiction. *The American Journal of Drug and …*, *13*(3), 231–251. http://doi.org/10.3109/00952998709001512

Hser, Y. I., Hoffman, V., Grella, C. E., & Anglin, M. D. (2001). A 33-year follow-up of narcotics addicts. *Archives of General Psychiatry*, *58*(5), 503–508.

Hurwitz, H., & De, S. C. (1958). Studies in light reinforced behavior: II. Effect of food deprivation and stress. *Psychological Reports*, *5*(3), 355. http://doi.org/10.2466/PR0.5.3.355-356

Hyman, S. E., Malenka, R. C., & Nestler, E. J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. *Annual Review of Neuroscience*, *29*(1), 565–598. http://doi.org/10.1146/annurev.neuro.29.051605.113009

Ivezaj, V., Saules, K. K., & Wiedemann, A. A. (2012). "I Didn't See This Coming.": Why Are Postbariatric Patients in Substance Abuse Treatment? Patients' Perceptions of Etiology and Future Recommendations. *Obesity Surgery*, *22*(8), 1308–1314. http://doi.org/10.1007/s11695-012-0668-2

Jackson, L. R., Robinson, T. E., & Becker, J. B. (2006). Sex differences and hormonal influences on acquisition of cocaine self-administration in rats. *Neuropsychopharmacology*, *31*(1), 129–138. http://doi.org/10.1038/sj.npp.1300778

Jacobs, E. H., Wardeh, G., Smit, A. B., & Schoffelmeer, A. N. M. (2005). Morphine causes a delayed increase in glutamate receptor functioning in the nucleus accumbens core. *European Journal of Pharmacology*, *511*(1), 27–30. http://doi.org/10.1016/j.ejphar.2005.02.009

Johnson, P. M., & Kenny, P. J. (2010). Addiction-like reward dysfunction and compulsive eating in obese rats: Role for dopamine D2 receptors. *Nature Neuroscience*, *13*(8), 1033–1033. http://doi.org/10.1038/nn0810-1033c

Jonas, J. M., Gold, M. S., Sweeney, D., & Pottash, A. L. (1987). Eating disorders and cocaine abuse: a survey of 259 cocaine abusers. *The Journal of Clinical Psychiatry*, *48*(2), 47–50. http://doi.org/10.1523/JNEUROSCI.4072-10.2010

Jones, S., & Bonci, A. (2005). Synaptic plasticity and drug addiction. *Current Opinion in Pharmacology*, *5*(1), 20–25. http://doi.org/10.1016/j.coph.2004.08.011

Jung, C., Rabinowitsch, A., Lee, W. T., Zheng, D., de Vaca, S. C., & Carr, K. D. (2016). Effects of food restriction on expression of place conditioning and biochemical correlates in rat nucleus accumbens. *Psychopharmacology*, *233*(17), 3161–3172. http://doi.org/10.1007/s00213-016-4360-4

Kaiser, S. K., Prendergast, K., & Editor, T. J. R. M. (2009). Nutritional Links to Substance Abuse Recovery. *Journal of Addictions Nursing*. http://doi.org/10.1080/10884600802305935;issue:issue:10.1080/ijan20.v019.i03;page:string:Article/Chapter

Kalivas, P. W. (2009). The glutamate homeostasishypothesis of addiction. *Nature Publishing Group*, *10*(8), 561–572. http://doi.org/10.1038/nrn2515

Kalivas, P. W., & Duffy, P. (1998). Repeated cocaine administration alters extracellular glutamate in the ventral tegmental area. *Journal of Neurochemistry*.

Kalivas, P. W., & Volkow, N. D. (2005). The Neural Basis of Addiction: A Pathology of Motivation and Choice. *American Journal of Psychiatry*, *162*(8), 1403–1413. http://doi.org/10.1176/appi.ajp.162.8.1403

Kalivas, P. W., LaLumiere, R. T., Knackstedt, L., & Shen, H. (2009). Glutamate transmission in addiction. *Neuropharmacology*, *56*, 169–173. http://doi.org/10.1016/j.neuropharm.2008.07.011

Kandel, D., & Kandel, E. (2015). The Gateway Hypothesis of substance abuse: developmental, biological and societal perspectives. *Acta Paediatrica (Oslo, Norway : 1992)*, *104*(2), 130–137. http://doi.org/10.1111/apa.12851

Katz, J. L., & Higgins, S. T. (2003). The validity of the reinstatement model of craving and relapse to drug use. *Psychopharmacology*, *168*(1-2), 21–30. http://doi.org/10.1007/s00213-003-1441-y

Keller, K. L., Vollrath-Smith, F. R., Jafari, M., & Ikemoto, S. (2014). Synergistic interaction between caloric restriction and amphetamine in food-unrelated approach behavior of rats. *Psychopharmacology*, *231*(5), 825–840. http://doi.org/10.1007/s00213-013-3300-9

Kelley, A. E., Baldo, B. A., Pratt, W. E., & Will, M. J. (2005). Corticostriatal-hypothalamic circuitry and food motivation: integration of energy, action and reward. *Physiology & Behavior*, *86*(5), 773–795. http://doi.org/10.1016/j.physbeh.2005.08.066

Kelz, M. B., Chen, J., Carlezon, W. A., Whisler, K., Gilden, L., Beckmann, A. M., et al. (1999). Expression of the transcription factor |[Delta]|FosB in the brain controls sensitivity to cocaine. *Nature*, *401*(6750), 272–276. http://doi.org/10.1038/45790

Kennedy, A. P., Epstein, D. H., Phillips, K. A., & Preston, K. L. (2013). Sex differences in cocaine/heroin users: drug-use triggers and craving in daily life. *Drug and Alcohol Dependence*, *132*(1-2), 29–37. http://doi.org/10.1016/j.drugalcdep.2012.12.025

Kenny, P. J. (2011a). Common cellular and molecular mechanisms in obesity and drug addiction. *Nature Reviews Neuroscience*, *12*(11), 638–651. http://doi.org/10.1038/nrn3105

Kenny, P. J. (2011b). Reward Mechanisms in Obesity: New Insights and Future Directions. *Neuron*, *69*(4), 664–679. http://doi.org/10.1016/j.neuron.2011.02.016

Kerstetter, K. A., Aguilar, V. R., Parrish, A. B., & Kippin, T. E. (2008). Protracted time-dependent increases in cocaine-seeking behavior during cocaine withdrawal in female relative to male rats. *Psychopharmacology*, *198*(1), 63–75. http://doi.org/10.1007/s00213-008-1089-8

Kippin, T. E., Fuchs, R. A., Mehta, R. H., Case, J. M., Parker, M. P., Bimonte-Nelson, H. A., & See, R. E. (2005). Potentiation of cocaine-primed reinstatement of drug seeking in female rats during estrus. *Psychopharmacology*, *182*(2), 245–252. http://doi.org/10.1007/s00213-005-0071-y

Kourrich, S., Rothwell, P. E., Klug, J. R., & Thomas, M. J. (2007). Cocaine experience controls bidirectional synaptic plasticity in the nucleus accumbens. *The Journal of Neuroscience :*

the Official Journal of the Society for Neuroscience, *27*(30), 7921–7928. http://doi.org/10.1523/JNEUROSCI.1859-07.2007

Krahn, D., Kurth, C., Demitrack, M., & Drewnowski, A. (1992). The relationship of dieting severity and bulimic behaviors to alcohol and other drug use in young women. *Journal of Substance Abuse*, *4*(4), 341–353.

Lacy, R. T., Strickland, J. C., Feinstein, M. A., Robinson, A. M., & Smith, M. A. (2016). The effects of sex, estrous cycle, and social contact on cocaine and heroin self-administration in rats. *Psychopharmacology*, *233*(17), 3201–3210. http://doi.org/10.1007/s00213-016-4368-9

LaLumiere, R. T., & Kalivas, P. W. (2008). Glutamate Release in the Nucleus Accumbens Core Is Necessary for Heroin Seeking. *Journal of Neuroscience*, *28*(12), 3170–3177. http://doi.org/10.1523/JNEUROSCI.5129-07.2008

Lancaster, F. E., & Spiegel, K. S. (1992). Sex differences in pattern of drinking. *Alcohol*, *9*(5), 415–420.

Larson, E. B., Roth, M. E., Anker, J. J., & Carroll, M. E. (2005). Effect of short- vs. long-term estrogen on reinstatement of cocaine-seeking behavior in female rats. *Pharmacology, Biochemistry and Behavior*, *82*(1), 98–108. http://doi.org/10.1016/j.pbb.2005.07.015

Lea, P. M., & Faden, A. I. (2006). Metabotropic glutamate receptor subtype 5 antagonists MPEP and MTEP. *CNS Drug Reviews*, *12*(2), 149–166. http://doi.org/10.1111/j.1527-3458.2006.00149.x

LeBlanc, K. H., Maidment, N. T., & Ostlund, S. B. (2014). Impact of repeated intravenous cocaine administration on incentive motivation depends on mode of drug delivery. *Addiction Biology*, *19*(6), 965–971. http://doi.org/10.1111/adb.12063

LeBlanc, K. H., Ostlund, S. B., & Maidment, N. T. (2012). Pavlovian-to-instrumental transfer in cocaine seeking rats. *Behavioral Neuroscience*, *126*(5), 681–689. http://doi.org/10.1037/a0029534

Lee, H.-K., Takamiya, K., Han, J.-S., Man, H., Kim, C.-H., Rumbaugh, G., et al. (2003). Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell*, *112*(5), 631–643.

Lee, J. L. C., Milton, A. L., & Everitt, B. J. (2006). Cue-induced cocaine seeking and relapse are reduced by disruption of drug memory reconsolidation. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *26*(22), 5881–5887. http://doi.org/10.1523/JNEUROSCI.0323-06.2006

Lee, R. S., Criado, J. R., Koob, G. F., & Henriksen, S. J. (1999). Cellular responses of nucleus accumbens neurons to opiate-seeking behavior: I. Sustained responding during heroin self-administration. *Synapse*, *33*(1), 49–58. http://doi.org/10.1002/(SICI)1098-2396(199907)33:1<49::AID-SYN5>3.0.CO;2-O

Lemieux, C., Phaneuf, D., Labrie, F., Giguère, V., Richard, D., & Deshaies, Y. (2005). Estrogen receptor alpha-mediated adiposity-lowering and hypocholesterolemic actions of the selective estrogen receptor modulator acolbifene. *International Journal of Obesity (2005)*, *29*(10), 1236–1244. http://doi.org/10.1038/sj.ijo.0803014

Levine, A. S., & Billington, C. J. (2004). Opioids as agents of reward-related feeding: a consideration of the evidence. *Physiology & Behavior*, *82*(1), 57–61. http://doi.org/10.1016/j.physbeh.2004.04.032

Li, N., He, S., Parrish, C., & Delich, J. (2003). Differences in morphine and cocaine reinforcement under fixed and progressive ratio schedules; effects of extinction, reacquisition and schedule design. *Behavioural* ….

Li, X., Xi, Z.-X., & Markou, A. (2013). Metabotropic glutamate 7 (mGlu7) receptor: a target for medication development for the treatment of cocaine dependence. *Neuropharmacology*, *66*, 12–23. http://doi.org/10.1016/j.neuropharm.2012.04.010

Lou, Z.-Z., Chen, L.-H., Liu, H.-F., Ruan, L.-M., & Zhou, W.-H. (2014). Blockade of mGluR5 in the nucleus accumbens shell but not core attenuates heroin seeking behavior in rats. *Acta Pharmacologica Sinica*, *35*(12), 1485–1492. http://doi.org/10.1038/aps.2014.93

Loweth, J. A., Tseng, K. Y., & Wolf, M. E. (2014). Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. *Neuropharmacology*, *76*, 287–300. http://doi.org/10.1016/j.neuropharm.2013.04.061

Lu, L., Shepard, J. D., Scott Hall, F., & Shaham, Y. (2003). Effect of environmental stressors on opiate and psychostimulant reinforcement, reinstatement and discrimination in rats: a

review. *Neuroscience and Biobehavioral Reviews*, *27*(5), 457–491. http://doi.org/10.1016/S0149-7634(03)00073-3

Lubow, R. E. (1973). Latent inhibition. *Psychological Bulletin*, *79*(6), 398–407. http://doi.org/10.1037/h0034425

Lutter, M., & Nestler, E. J. (2009). Homeostatic and hedonic signals interact in the regulation of food intake. *Journal of Nutrition*, *139*(3), 629–632. http://doi.org/10.3945/jn.108.097618

Lynch, W. J. (2017). Modeling the development of drug addiction in male and female animals. *Pharmacology, Biochemistry and Behavior*. http://doi.org/10.1016/j.pbb.2017.06.006

Lynch, W. J., & Carroll, M. E. (1999). Sex differences in the acquisition of intravenously self-administered cocaine and heroin in rats. *Psychopharmacology*, *144*(1), 77–82.

Lynch, W. J., Roth, M. E., & Carroll, M. E. (2002). Biological basis of sex differences in drug abuse: preclinical and clinical studies. *Psychopharmacology*, *164*(2), 121–137. http://doi.org/10.1007/s00213-002-1183-2

Lynch, W. J., Roth, M. E., Mickelberg, J. L., & Carroll, M. E. (2001). Role of estrogen in the acquisition of intravenously self-administered cocaine in female rats. *Pharmacology, Biochemistry and Behavior*, *68*(4), 641–646. http://doi.org/10.1016/S0091-3057(01)00455-5

Lynch, W. J., & Taylor, J. R. (2005). Decreased Motivation Following Cocaine Self-Administration Under Extended Access Conditions: Effects of Sex and Ovarian Hormones - ProQuest. *Neuropsychopharmacology*.

Ma, Y.-Y., Lee, B. R., Wang, X., Guo, C., Liu, L., Cui, R., et al. (2014). Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. *Neuron*, *83*(6), 1453–1467. http://doi.org/10.1016/j.neuron.2014.08.023

Macenski, M. J., & Meisch, R. A. (1999). Cocaine self-administration under conditions of restricted and unrestricted food access. *Experimental and Clinical …*, *7*(4), 324–337. http://doi.org/10.1037/1064-1297.7.4.324

Mackintosh, N. J. (1974). The psychology of animal learning. Academic Press.

Madsen, H. B., & Ahmed, S. H. (2015). Drug versus sweet reward: greater attraction to and preference for sweet versus drug cues. *Addiction Biology*, *20*(3), 433–444. http://doi.org/10.1111/adb.12134

Maehira, Y., Chowdhury, E. I., Reza, M., Drahozal, R., Gayen, T. K., Masud, I., et al. (2013). Factors associated with relapse into drug use among male and female attendees of a three-month drug detoxification-rehabilitation programme in Dhaka, Bangladesh: a prospective cohort study. *Harm Reduction Journal*, *10*(1), 14. http://doi.org/10.1186/1477-7517-10-14

Mammen, A. L., Kameyama, K., & Roche, K. W. (1997). Phosphorylation of the α-amino-3-hydroxy-5-methylisoxazole4-propionic acid receptor GluR1 subunit by calcium/calmodulin-dependent kinase II. *Journal of Biological …*, *272*(51), 32528–32533. http://doi.org/10.1074/jbc.272.51.32528

Marinelli, M., Le Moal, M., & Piazza, P. V. (1996). Acute pharmacological blockade of corticosterone secretion reverses food restriction-induced sensitization of the locomotor response to cocaine. *Brain Research*, *724*(2), 251–255.

Marks-Kaufman, R. (1982). Increased fat consumption induced by morphine administration in rats. *Pharmacology, Biochemistry and Behavior*. http://doi.org/10.1016/0091-3057(82)90051-X

Marks-Kaufman, R., & Kanarek, R. B. (1990). Diet selection following a chronic morphine and naloxone regimen. *Pharmacology, Biochemistry and Behavior*. http://doi.org/10.1016/0091-3057(90)90305-2

Marusich, J. A., Beckmann, J. S., Gipson, C. D., & Bardo, M. T. (2011). Cue Effects on Methylphenidate Self-Administration in Rats. *Behavioural Pharmacology*, *22*(7), 714–717. http://doi.org/10.1097/FBP.0b013e32834afed1

Matsubara, M., Sakata, I., Wada, R., Yamazaki, M., Inoue, K., & Sakai, T. (2004). Estrogen modulates ghrelin expression in the female rat stomach. *Peptides*, *25*(2), 289–297. http://doi.org/10.1016/j.peptides.2003.12.020

Mavrikaki, M., Pravetoni, M., Page, S., Potter, D., & Chartoff, E. (2017). Oxycodone self-administration in male and female rats. *Psychopharmacology*, *234*(6), 977–987. http://doi.org/10.1007/s00213-017-4536-6

Mayo, L. M., Fraser, D., Childs, E., Momenan, R., Hommer, D. W., de Wit, H., & Heilig, M. (2013). Conditioned preference to a methamphetamine-associated contextual cue in humans. *Neuropsychopharmacology*, *38*(6), 921–929. http://doi.org/10.1038/npp.2013.3

McCutcheon, J. E., Wang, X., Tseng, K. Y., Wolf, M. E., & Marinelli, M. (2011). Calcium-Permeable AMPA Receptors Are Present in Nucleus Accumbens Synapses after Prolonged Withdrawal from Cocaine Self-Administration But Not Experimenter-Administered Cocaine. *Journal of Neuroscience*, *31*(15), 5737–5743. http://doi.org/10.1523/JNEUROSCI.0350-11.2011

McKay, J. R., Franklin, T. R., Patapis, N., & Lynch, K. G. (2006). Conceptual, methodological, and analytical issues in the study of relapse. *Clinical Psychology Review*, *26*(2), 109–127. http://doi.org/10.1016/j.cpr.2005.11.002

Micevych, P., Soma, K. K., & Sinchak, K. (2008). Neuroprogesterone: Key to estrogen positive feedback? *Brain Research Reviews*, *57*(2), 470–480. http://doi.org/10.1016/j.brainresrev.2007.06.009

Milton, A. L., & Everitt, B. J. (2010). The psychological and neurochemical mechanisms of drug memory reconsolidation: implications for the treatment of addiction. *European Journal of Neuroscience*, *31*(12), 2308–2319. http://doi.org/10.1111/j.1460-9568.2010.07249.x

Milton, A. L., & Everitt, B. J. (2012). The persistence of maladaptive memory: Addiction, drug memories and anti-relapse treatments. *Neuroscience and Biobehavioral Reviews*, *36*(4), 1119–1139. http://doi.org/10.1016/j.neubiorev.2012.01.002

Minervini, V., Rowland, N. E., Robertson, K. L., & Foster, T. C. (2015). Role of estrogen receptor-α on food demand elasticity. *Journal of the Experimental Analysis of Behavior*, *103*(3), 553–561. http://doi.org/10.1002/jeab.149

Mora, S., Dussaubat, N., & Díaz-Véliz, G. (1996). Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. *Psychoneuroendocrinology*, *21*(7), 609–620. http://doi.org/10.1016/S0306-4530(96)00015-7

Morris, J. B., Crowder, W. F., & Crowder, T. H. (1961). Stimulus variations and weak-light reinforcement. *Psychological Reports*. http://doi.org/10.2466/pr0.1961.8.2.290

Mucha, R. F., Geier, A., Stuhlinger, M., & Mundle, G. (2000). Appetitive effects of drug cues modelled by pictures of the intake ritual: generality of cue-modulated startle examined with inpatient alcoholics. *Psychopharmacology*, *151*(4), 428–432. http://doi.org/10.1007/s002130000508

Mucha, R. F., Pauli, P., & Angrilli, A. (1998). Conditioned responses elicited by experimentally produced cues for smoking. *Canadian Journal of Physiology and Pharmacology*, *76*(3), 259–268.

Myers, M. G., Jr, Münzberg, H., Leinninger, G. M., & Leshan, R. L. (2009). The Geometry of Leptin Action in the Brain: More Complicated Than a Simple ARC. *Cell Metabolism*, *9*(2), 117–123. http://doi.org/10.1016/j.cmet.2008.12.001

Nakazato, M., Murakami, N., Date, Y., & Kojima, M. (2001). A role for ghrelin in the central regulation of feeding - ProQuest. *Nature*.

Neale, J., Nettleton, S., Pickering, L., & Fischer, J. (2012). Eating patterns among heroin users: a qualitative study with implications for nutritional interventions. *Addiction*, *107*(3), 635–641. http://doi.org/10.1111/j.1360-0443.2011.03660.x

Nestler, E. J. (2005). Is there a common molecular pathway for addiction? *Nature Neuroscience*. http://doi.org/10.1038/nn1578

Nestler, E. J. (2008). Review. Transcriptional mechanisms of addiction: role of DeltaFosB. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *363*(1507), 3245–3255. http://doi.org/10.1098/rstb.2008.0067

Noble, C., & McCombie, L. (1997). Nutritional considerations in intravenous drug misusers: a review of the literature and current issues for dietitians. *Journal of Human Nutrition and Dietetics*, *10*(3), 181–191. http://doi.org/10.1046/j.1365-277X.1997.00051.x

Nolan, L. J., & Scagnelli, L. M. (2007). Preference for sweet foods and higher body mass index in patients being treated in long-term methadone maintenance. *Substance Use & Misuse*, *42*(10), 1555–1566. http://doi.org/10.1080/10826080701517727

Oh, M. C., Derkach, V. A., Guire, E. S., & Soderling, T. R. (2006). Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for

long-term potentiation. *The Journal of Biological Chemistry*, *281*(2), 752–758. http://doi.org/10.1074/jbc.M509677200

Ohlsson, C., Hellberg, N., Parini, P., Vidal, O., Bohlooly, M., Rudling, M., et al. (2000). Obesity and Disturbed Lipoprotein Profile in Estrogen Receptor-α-Deficient Male Mice. *Biochemical and Biophysical Research Communications*, *278*(3), 640–645. http://doi.org/10.1006/bbrc.2000.3827

Olsen, C. M., & Winder, D. G. (2012). Stimulus dynamics increase the self-administration of compound visual and auditory stimuli. *Neuroscience Letters*, *511*(1), 8–11. http://doi.org/10.1016/j.neulet.2011.12.068

Orain-Pelissolo, S., Grillon, C., Perez-Diaz, F., & Jouvent, R. (2004). Lack of startle modulation by smoking cues in smokers. *Psychopharmacology*, *173*(1-2), 160–166. http://doi.org/10.1007/s00213-003-1715-4

Ostlund, S. B., & Balleine, B. W. (2008). On habits and addiction: An associative analysis of compulsive drug seeking. *Drug Discovery Today: Disease Models*, *5*(4), 235–245. http://doi.org/10.1016/j.ddmod.2009.07.004

Ouyang, J., Carcea, I., Schiavo, J. K., Jones, K. T., Rabinowitsch, A., Kolaric, R., et al. (2017). Food restriction induces synaptic incorporation of calcium-permeable AMPA receptors in nucleus accumbens. *European Journal of Neuroscience*, *45*(6), 826–836. http://doi.org/10.1111/ejn.13528

Palmatier, M. I., Evans-Martin, F. F., Hoffman, A., Caggiula, A. R., Chaudhri, N., Donny, E. C., et al. (2006). Dissociating the primary reinforcing and reinforcement-enhancing effects of nicotine using a rat self-administration paradigm with concurrently available drug and environmental reinforcers. *Psychopharmacology*, *184*(3-4), 391–400. http://doi.org/10.1007/s00213-005-0183-4

Palmatier, M. I., Liu, X., Matteson, G. L., Donny, E. C., Caggiula, A. R., & Sved, A. F. (2007). Conditioned reinforcement in rats established with self-administered nicotine and enhanced by noncontingent nicotine. *Psychopharmacology*, *195*(2), 235–243. http://doi.org/10.1007/s00213-007-0897-6

Paxinos, G., & Watson, C. (2005). The Rat Brain (5 ed.). Elsevier.

Peng, X. X., Lister, A., Rabinowitsch, A., Kolaric, R., Cabeza de Vaca, S., Ziff, E. B., & Carr, K. D. (2015). Episodic sucrose intake during food restriction increases synaptic abundance of AMPA receptors in nucleus accumbens and augments intake of sucrose following restoration of ad libitum feeding. *Neuroscience*, *295*, 58–71. http://doi.org/10.1016/j.neuroscience.2015.03.025

Peng, X.-X., Cabeza de Vaca, S., Ziff, E. B., & Carr, K. D. (2014). Involvement of nucleus accumbens AMPA receptor trafficking in augmentation of D- amphetamine reward in food-restricted rats. *Psychopharmacology*, *231*(15), 3055–3063. http://doi.org/10.1007/s00213-014-3476-7

Perrotti, L. I., Weaver, R. R., Robison, B., Renthal, W., Maze, I., Yazdani, S., et al. (2008). Distinct patterns of DeltaFosB induction in brain by drugs of abuse. *Synapse*, *62*(5), 358–369. http://doi.org/10.1002/syn.20500

Peters, J., & De Vries, T. J. (2014). Pavlovian conditioned approach, extinction, and spontaneous recovery to an audiovisual cue paired with an intravenous heroin infusion. *Psychopharmacology*, *231*(2), 447–453. http://doi.org/10.1007/s00213-013-3258-7

Piazza, P. V., & Le Moal, M. (1998). The role of stress in drug self-administration. *Trends in Pharmacological Sciences*, *19*(2), 67–74. http://doi.org/10.1016/S0165-6147(97)01115-2

Pickens, C. L., Airavaara, M., Theberge, F., Fanous, S., Hope, B. T., & Shaham, Y. (2011). Neurobiology of the incubation of drug craving. *Trends in Neurosciences*, *34*(8), 411–420. http://doi.org/10.1016/j.tins.2011.06.001

Pierce, R. C., Meil, W. M., & Kalivas, P. W. (1997). The NMDA antagonist, dizocilpine, enhances cocaine reinforcement without influencing mesoaccumbens dopamine transmission. *Psychopharmacology*, *133*(2), 188–195.

Ping, A., Xi, J., Prasad, B. M., Wang, M.-H., & Kruzich, P. J. (2008). Contributions of nucleus accumbens core and shell GluR1 containing AMPA receptors in AMPA- and cocaine-primed reinstatement of cocaine-seeking behavior. *Brain Research*, *1215*, 173–182. http://doi.org/10.1016/j.brainres.2008.03.088

Pisetsky, E. M., Chao, Y. M., Dierker, L. C., May, A. M., & Striegel-Moore, R. H. (2008). Disordered eating and substance use in high-school students: results from the Youth Risk Behavior

Surveillance System. *International Journal of Eating Disorders*, *41*(5), 464–470. http://doi.org/10.1002/eat.20520

Pomierny-Chamioło, L., Rup, K., Pomierny, B., Niedzielska, E., Kalivas, P. W., & Filip, M. (2014). Metabotropic glutamatergic receptors and their ligands in drug addiction. *Pharmacology & Therapeutics*, *142*(3), 281–305. http://doi.org/10.1016/j.pharmthera.2013.12.012

Preston, K. L., Kowalczyk, W. J., Phillips, K. A., Jobes, M. L., Vahabzadeh, M., Lin, J.-L., et al. (2018). Exacerbated Craving in the Presence of Stress and Drug Cues in Drug-Dependent Patients. *Neuropsychopharmacology*, *43*(4), 859–867. http://doi.org/10.1038/npp.2017.275

Purgianto, A., Scheyer, A. F., Loweth, J. A., Ford, K. A., Tseng, K. Y., & Wolf, M. E. (2013). Different Adaptations in AMPA Receptor Transmission in the Nucleus Accumbens after Short vs Long Access Cocaine Self-Administration Regimens. *Neuropsychopharmacology*, *38*(9), 1789–1797. http://doi.org/10.1038/npp.2013.78

Quintero, E., Díaz, E., Vargas, J. P., Schmajuk, N., López, J. C., & la Casa, De, L. G. (2011). Effects of context novelty vs. familiarity on latent inhibition with a conditioned taste aversion procedure. *Behavioural Processes*, *86*(2), 242–249. http://doi.org/10.1016/j.beproc.2010.12.011

Randall, P. A., Pardo, M., Nunes, E. J., Cruz, L. L., Vemuri, V. K., Makriyannis, A., et al. (2012). Dopaminergic Modulation of Effort-Related Choice Behavior as Assessed by a Progressive Ratio Chow Feeding Choice Task: Pharmacological Studies and the Role of Individual Differences. *PLoS ONE*, *7*(10), e47934. http://doi.org/10.1371/journal.pone.0047934

Reimers, J. M., Milovanovic, M., & Wolf, M. E. (2011). Quantitative analysis of AMPA receptor subunit composition in addiction-related brain regions. *Brain Research*, *1367*, 223–233. http://doi.org/10.1016/j.brainres.2010.10.016

Rescorla, R. A. (1994). Control of instrumental performance by Pavlovian and instrumental stimuli. *Journal of Experimental Psychology. Animal Behavior Processes*, *20*(1), 44–50.

Rescorla, R. A., & Solomon, R. L. (1967). Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychological Review*, *74*(3), 151–182.

Richardson, N. R., & Roberts, D. C. (1996). Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *Journal of Neuroscience Methods*, *66*(1), 1–11.

Robbins, S. J., Ehrman, R. N., Childress, A. R., & O'Brien, C. P. (1999). Comparing levels of cocaine cue reactivity in male and female outpatients. *Drug and Alcohol Dependence*, *53*(3), 223–230.

Roberts, D. C. S., Bennett, S. A. L., & Vickers, G. J. (1989a). The estrous cycle affects cocaine self-administration on a progressive ratio schedule in rats. *Psychopharmacology*, *98*(3), 408–411. http://doi.org/10.1007/BF00451696

Roberts, D. C., Bennett, S. A., & Vickers, G. J. (1989b). The estrous cycle affects cocaine self-administration on a progressive ratio schedule in rats. *Psychopharmacology*, *98*(3), 408–411.

Robins, L. N., & Regier, D. A. (1991). Psychiatric disorders in America: the epidemiologic catchment area study.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Research Reviews*, *18*(3), 247–291. http://doi.org/10.1016/0165-0173(93)90013-P

Robinson, T. E., & Berridge, K. C. (2008). The incentive sensitization theory of addiction: some current issues. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*(1507), 3137–3146. http://doi.org/10.1098/rstb.2008.0093

Robinson, T. E., Gorny, G., Savage, V. R., & Kolb, B. (2002). Widespread but regionally specific effects of experimenter- versus self-administered morphine on dendritic spines in the nucleus accumbens, hippocampus, and neocortex of adult rats. *Synapse*, *46*(4), 271–279. http://doi.org/10.1002/syn.10146

Robinson, T. E., Yager, L. M., Cogan, E. S., & Saunders, B. T. (2014). On the motivational properties of reward cues: Individual differences. *Neuropharmacology*, *76 Pt B*, 450–459. http://doi.org/10.1016/j.neuropharm.2013.05.040

Rocha, M., Bing, C., Williams, G., & Puerta, M. (2004). Physiologic estradiol levels enhance hypothalamic expression of the long form of the leptin receptor in intact rats. *The Journal of Nutritional Biochemistry*, *15*(6), 328–334. http://doi.org/10.1016/j.jnutbio.2004.01.003

Roesch, D. M. (2006). Effects of selective estrogen receptor agonists on food intake and body weight gain in rats. *Physiology & Behavior*, *87*(1), 39–44. http://doi.org/10.1016/j.physbeh.2005.08.035

Rosse, R., Deutsch, S., & Chilton, M. (2005). Cocaine addicts prone to cocaine-induced psychosis have lower body mass index than cocaine addicts resistant to cocaine-induced psychosis--Implications for the cocaine model of psychosis proneness. *The Israel Journal of Psychiatry and Related Sciences*, *42*(1), 45–50.

Roth, M. E., Casimir, A. G., & Carroll, M. E. (2002). Influence of estrogen in the acquisition of intravenously self-administered heroin in female rats. *Pharmacology, Biochemistry and Behavior*, *72*(1-2), 313–318.

Roth, M. E., Cosgrove, K. P., & Carroll, M. E. (2004). Sex differences in the vulnerability to drug abuse: a review of preclinical studies. *Neuroscience and Biobehavioral Reviews*, *28*(6), 533–546. http://doi.org/10.1016/j.neubiorev.2004.08.001

Rudd, R. A., Aleshire, N., Zibbell, J. E., & Gladden, R. M. (2016). Increases in Drug and Opioid Overdose Deaths--United States, 2000-2014. *MMWR. Morbidity and Mortality Weekly Report*, *64*(50-51), 1378–1382. http://doi.org/10.15585/mmwr.mm6450a3

Salamone, J. D., & Correa, M. (2009). Dopamine/adenosine interactions involved in effort-related aspects of food motivation. *Appetite*, *53*(3), 422–425. http://doi.org/10.1016/j.appet.2009.07.018

Salamone, J. D., Kurth, P. A., McCullough, L. D., Sokolowski, J. D., & Cousins, M. S. (1993). The role of brain dopamine in response initiation: effects of haloperidol and regionally specific dopamine depletions on the local rate of instrumental responding. *Brain Research*, *628*(1-2), 218–226. http://doi.org/10.1016/0006-8993(93)90958-P

Samaha, A.-N., Minogianis, E.-A., & Nachar, W. (2011). Cues Paired with either Rapid or Slower Self-Administered Cocaine Injections Acquire Similar Conditioned Rewarding Properties. *PLoS ONE*, *6*(10), e26481. http://doi.org/10.1371/journal.pone.0026481

Sanchis Segura, C., & Becker, J. B. (2016). Why we should consider sex (and study sex differences) in addiction research. *Addiction Biology*, *21*(5), 995–1006. http://doi.org/10.1111/adb.12382

Santen, F. J., Sofsky, J., Bilic, N., & Lippert, R. (1975). Mechanism of action of narcotics in the production of menstrual dysfunction in women. *Fertility and Sterility*, *26*(6), 538–548.

Santolaria-Fernández, F. (1995). Nutritional assessment of drug addicts. *Drug and Alcohol Dependence*, *38*(1), 11–18. http://doi.org/10.1016/0376-8716(94)01088-3

Santollo, J., Wiley, M. D., & Eckel, L. A. (2007). Acute activation of ERα decreases food intake, meal size, and body weight in ovariectomized rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *293*(6), R2194–R2201. http://doi.org/10.1152/ajpregu.00385.2007

Santos, S. D., Carvalho, A. L., Caldeira, M. V., & Duarte, C. B. (2009). Regulation of AMPA receptors and synaptic plasticity. *Nsc*, *158*(1), 105–125. http://doi.org/10.1016/j.neuroscience.2008.02.037

Satoh, M., Zieglgänsberger, W., & Herz, A. (1976). Supersensitivity of cortical neurones of the rat to acetylcholine and L-glutamate following chronic morphine treatment. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *293*(1), 101–103.

Saunders, B. T., & Robinson, T. E. (2010). A cocaine cue acts as an incentive stimulus in some but not others: implications for addiction. *Biological Psychiatry*, *67*(8), 730–736. http://doi.org/10.1016/j.biopsych.2009.11.015

Saunders, B. T., & Robinson, T. E. (2012). The role of dopamine in the accumbens core in the expression of Pavlovian-conditioned responses. *European Journal of Neuroscience*, *36*(4), 2521–2532. http://doi.org/10.1111/j.1460-9568.2012.08217.x

Schoepp, D. D. (2001). Unveiling the Functions of Presynaptic Metabotropic Glutamate Receptors in the Central Nervous System. *The Journal of Pharmacology and Experimental Therapeutics*, *299*(1), 12–20. http://doi.org/10.1016/S0165-0173(98)00050-2

Schramm-Sapyta, N. L., Olsen, C. M., & Winder, D. G. (2006). Cocaine self-administration reduces excitatory responses in the mouse nucleus accumbens shell. *Neuropsychopharmacology*, *31*(7), 1444–1451. http://doi.org/10.1038/sj.npp.1300918

Sedki, F., Abbas, Z., Angelis, S., Martin, J., D'Cunha, T., & Shalev, U. (2013a). Is it stress? The role of stress related systems in chronic food restriction-induced augmentation of heroin seeking in the rat., *7*, 98. http://doi.org/10.3389/fnins.2013.00098

Sedki, F., D'Cunha, T., & Shalev, U. (2013b). A procedure to study the effect of prolonged food restriction on heroin seeking in abstinent rats. *Journal of Visualized Experiments : JoVE*, (81), e50751. http://doi.org/10.3791/50751

Sedki, F., Gregory, J. G., Luminare, A., D'Cunha, T. M., & Shalev, U. (2015). Food restriction-induced augmentation of heroin seeking in female rats: manipulations of ovarian hormones. *Psychopharmacology*, *232*(20), 3773–3782. http://doi.org/10.1007/s00213-015-4037-4

See, R. E., Grimm, J. W., Kruzich, P. J., & Rustay, N. (1999). The importance of a compound stimulus in conditioned drug-seeking behavior following one week of extinction from self-administered cocaine in rats. *Drug and Alcohol Dependence*. http://doi.org/10.1016/S0376-8716(99)00043-5

Segal, E. F. (1959). Confirmation of a positive relation between deprivation and number of responses emitted for light reinforcement. *Journal of the Experimental Analysis of Behavior*, *2*(2), 165–169. http://doi.org/10.1901/jeab.1959.2-165

Sepulveda, M. J., Hernandez, L., Rada, P., Tucci, S., & Contreras, E. (1998). Effect of precipitated withdrawal on extracellular glutamate and aspartate in the nucleus accumbens of chronically morphine-treated rats: an in vivo microdialysis study. *Pharmacology, Biochemistry and Behavior*, *60*(1), 255–262.

Sepúlveda, J., Oliva, P., & Contreras, E. (2004). Neurochemical changes of the extracellular concentrations of glutamate and aspartate in the nucleus accumbens of rats after chronic administration of morphine. *European Journal of Pharmacology*, *483*(2-3), 249–258.

Seth, P., Rudd, R. A., Noonan, R. K., & Haegerich, T. M. (2018). Quantifying the Epidemic of Prescription Opioid Overdose Deaths. *American Journal of Public Health*, *108*(4), 500–502. http://doi.org/10.2105/AJPH.2017.304265

Shaham, Y., & Stewart, J. (1995). Effects of restraint stress and intra-ventral tegmental area injections of morphine and methyl naltrexone on the discriminative stimulus effects of

heroin in the rat. *Pharmacology, Biochemistry and Behavior*, *51*(2-3), 491–498. http://doi.org/10.1016/0091-3057(95)00015-O

Shaham, Y., Rajabi, H., & Stewart, J. (1996). Relapse to heroin-seeking in rats under opioid maintenance: the effects of stress, heroin priming, and withdrawal. *Journal of Neuroscience*, *16*(5), 1957–1963. http://doi.org/10.1523/JNEUROSCI.16-05-01957.1996

Shaham, Y., Shalev, U., Lu, L., de Wit, H., & Stewart, J. (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology*, *168*(1-2), 3–20. http://doi.org/10.1007/s00213-002-1224-x

Shalev, U. (2012). Chronic food restriction augments the reinstatement of extinguished heroin-seeking behavior in rats. *Addiction Biology*, *17*(4), 691–693. http://doi.org/10.1111/j.1369-1600.2010.00303.x

Shalev, U., Finnie, P. S., Quinn, T., Tobin, S., & Wahi, P. (2006). A role for corticotropin-releasing factor, but not corticosterone, in acute food-deprivation-induced reinstatement of heroin seeking in rats. *Psychopharmacology*, *187*(3), 376–384. http://doi.org/10.1007/s00213-006-0427-y

Shalev, U., Grimm, J. W., & Shaham, Y. (2002). Neurobiology of relapse to heroin and cocaine seeking: a review. *Pharmacological Reviews*, *54*(1), 1–42. Retrieved from http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11870259&retmode=ref&cmd=prlinks

Shen, H., & Kalivas, P. W. (2013). Reduced LTP and LTD in prefrontal cortex synapses in the nucleus accumbens after heroin self-administration. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, *16*(5), 1165–1167. http://doi.org/10.1017/S1461145712001071

Shen, H., Moussawi, K., Zhou, W., Toda, S., & Kalivas, P. W. (2011). Heroin relapse requires long-term potentiation-like plasticity mediated by NMDA2b-containing receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(48), 19407–19412. http://doi.org/10.1073/pnas.1112052108

Shen, H.-W., Toda, S., Moussawi, K., Bouknight, A., Zahm, D. S., & Kalivas, P. W. (2009). Altered dendritic spine plasticity in cocaine-withdrawn rats. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *29*(9), 2876–2884. http://doi.org/10.1523/JNEUROSCI.5638-08.2009

Shin, C. B., Templeton, T. J., Chiu, A. S., Kim, J., Gable, E. S., Vieira, P. A., et al. (2018). Endogenous glutamate within the prelimbic and infralimbic cortices regulates the incubation of cocaine-seeking in rats. *Neuropharmacology*, *128*, 293–300. http://doi.org/10.1016/j.neuropharm.2017.10.024

Shin, R., Cao, J., Webb, S. M., & Ikemoto, S. (2010). Amphetamine Administration into the Ventral Striatum Facilitates Behavioral Interaction with Unconditioned Visual Signals in Rats. *PLoS ONE*, *5*(1), e8741. http://doi.org/10.1371/journal.pone.0008741

Simon, G. E., Korff, Von, M., Saunders, K., Miglioretti, D. L., Crane, P. K., van Belle, G., & Kessler, R. C. (2006). Association between obesity and psychiatric disorders in the US adult population. *Archives of General Psychiatry*, *63*(7), 824–830. http://doi.org/10.1001/archpsyc.63.7.824

Singer, B. F., Fadanelli, M., Kawa, A. B., & Robinson, T. E. (2018). Are Cocaine-Seeking "Habits" Necessary for the Development of Addiction-Like Behavior in Rats? *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *38*(1), 60–73. http://doi.org/10.1523/JNEUROSCI.2458-17.2017

Sinha, R. (2001). How does stress increase risk of drug abuse and relapse? *Psychopharmacology*, *158*(4), 343–359. http://doi.org/10.1007/s002130100917

Sinha, R., Fox, H., Hong, K.-I., Sofuoglu, M., Morgan, P. T., & Bergquist, K. T. (2007). Sex steroid hormones, stress response, and drug craving in cocaine-dependent women: implications for relapse susceptibility. *Experimental and Clinical Psychopharmacology*, *15*(5), 445–452. http://doi.org/10.1037/1064-1297.15.5.445

Smith, R. C., & Donahoe, J. W. (1966). The effects of food deprivation on unreinforced and light-reinforced bar pressing. *The Journal of Genetic Psychology*.

Sofuoglu, M., Dudish-Poulsen, S., Nelson, D., Pentel, P. R., & Hatsukami, D. K. (1999). Sex and menstrual cycle differences in the subjective effects from smoked cocaine in humans. *Experimental and Clinical Psychopharmacology*, *7*(3), 274–283.

Spencer, S., Scofield, M., & Kalivas, P. W. (2016). The good and bad news about glutamate in drug addiction. *Journal of Psychopharmacology (Oxford, England)*. http://doi.org/10.1177/0269881116655248

Stephens, D. N., & Brown, G. (1999). Disruption of operant oral self-administration of ethanol, sucrose, and saccharin by the AMPA/kainate antagonist, NBQX, but not the AMPA antagonist, GYKI 52466. *Alcoholism: Clinical and Experimental Research*, *23*(12), 1914–1920.

Stewart, J. (1960). Reinforcing effects of light as a function of intensity and reinforcement schedule. *Journal of Comparative and Physiological Psychology*, *53*(2), 187–193. http://doi.org/10.1037/h0047315

Stewart, J. (2000). Pathways to relapse: the neurobiology of drug- and stress-induced relapse to drug-taking. *Journal of Psychiatry & Neuroscience*, *25*(2), 125–136.

Stewart, J., de Wit, H., & Eikelboom, R. (1984). Role of unconditioned and conditioned drug effects in the self-administration of opiates and stimulants. *Psychological Review*, *91*(2), 251–268.

Stewart, J., Woodside, B., & Shaham, Y. (1996). Ovarian hormones do not affect the initiation and maintenance of intravenous self-administration of heroin in the female rat. *Psychobiology*. http://doi.org/10.3758/BF03331967

Stice, E., Spoor, S., Bohon, C., & Small, D. M. (2008). Relation between obesity and blunted striatal response to food is moderated by TaqIA A1 allele. *Science*, *322*(5900), 449–452. http://doi.org/10.1126/science.1161550

Stuber, G. D., Evans, S. B., Higgins, M. S., Pu, Y., & Figlewicz, D. P. (2002). Food restriction modulates amphetamine-conditioned place preference and nucleus accumbens dopamine release in the rat. *Synapse*, *46*(2), 83–90. http://doi.org/10.1002/syn.10120

Stuber, G. D., Hnasko, T. S., Britt, J. P., Edwards, R. H., & Bonci, A. (2010). Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *The*

Journal of Neuroscience : the Official Journal of the Society for Neuroscience, *30*(24), 8229–8233. http://doi.org/10.1523/JNEUROSCI.1754-10.2010

Substance Abuse and Mental Health Services Administration. (2013). *The DAWN report: highlights of the 2011 Drug Abuse Warning Network (DAWN) findings on drug-related emergency department visits*.

(No. (SMA) 13-4760, DAWN Series D-39). *samhsa.gov*. Rockville, MD.

Substance Abuse and Mental Health Services Administration. (2014). *Treatment Episode Data Set (TEDS) 2002-2012*

(No. bhdid sERIES s-72, hhs, 14 ed.). *samhsa.gov*. Rockville, MD.

Sun, W., & Rebec, G. V. (2006). Repeated cocaine self-administration alters processing of cocaine-related information in rat prefrontal cortex. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *26*(30), 8004–8008. http://doi.org/10.1523/JNEUROSCI.1413-06.2006

Sun, X., Milovanovic, M., Zhao, Y., & Wolf, M. E. (2008). Acute and chronic dopamine receptor stimulation modulates AMPA receptor trafficking in nucleus accumbens neurons cocultured with prefrontal cortex neurons. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *28*(16), 4216–4230. http://doi.org/10.1523/JNEUROSCI.0258-08.2008

Tetrault, J. M., & Butner, J. L. (2015). Non-Medical Prescription Opioid Use and Prescription Opioid Use Disorder: A Review. *The Yale Journal of Biology and Medicine*, *88*(3), 227–233.

Thomas, M. J., Beurrier, C., Bonci, A., & Malenka, R. C. (2001). Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. *Nature Neuroscience*, *4*(12), 1217–1223. http://doi.org/10.1038/nn757

Tobin, S., & Shalev, U. (2012). Glutamatergic Receptor Expression Following Heroin-Self-Administartion and Acute Food Deprivation-Induced Resisntatement of Heroin Seeking. . *Unpublished Manuscript*.

Tobin, S., Sedki, F., Abbas, Z., & Shalev, U. (2013). Antagonism of the dopamine D1-like receptor in mesocorticolimbic nuclei attenuates acute food deprivation-induced

reinstatement of heroin seeking in rats. *European Journal of Neuroscience*, *37*(6), 972–981. http://doi.org/10.1111/ejn.12112

Tomiyama, A. J., Mann, T., Vinas, D., Hunger, J. M., DeJager, J., & Taylor, S. E. (2010). Low calorie dieting increases cortisol. *Psychosomatic Medicine*, *72*(4), 357–364. http://doi.org/10.1097/PSY.0b013e3181d9523c

Uslaner, J. M., Acerbo, M. J., Jones, S. A., & Robinson, T. E. (2006). The attribution of incentive salience to a stimulus that signals an intravenous injection of cocaine. *Behavioural Brain Research*, *169*(2), 320–324. http://doi.org/10.1016/j.bbr.2006.02.001

US Department of Justice National Drug Intelligence Center. (n.d.). *National Drug Threat Assessment*. National Drug Intelligence Center. Retrieved from https://www.justice.gov/archive/ndic/pubs44/44849/44849p.pdf

Valyear, M. D., Villaruel, F. R., & Chaudhri, N. (2017). Alcohol-seeking and relapse: A focus on incentive salience and contextual conditioning. *Behavioural Processes*, *141*(Pt 1), 26–32. http://doi.org/10.1016/j.beproc.2017.04.019

Van den Oever, M. C., Goriounova, N. A., Li, K. W., Van der Schors, R. C., Binnekade, R., Schoffelmeer, A. N. M., et al. (2008). Prefrontal cortex AMPA receptor plasticity is crucial for cue-induced relapse to heroin-seeking. *Nature Neuroscience*, *11*(9), 1053–1058. http://doi.org/10.1038/nn.2165

Van Etten, M. L., & Anthony, J. C. (2001). Male-female differences in transitions from first drug opportunity to first use: searching for subgroup variation by age, race, region, and urban status. *Journal of Women's Health & Gender-Based Medicine*, *10*(8), 797–804. http://doi.org/10.1089/15246090152636550

Van Etten, M. L., Neumark, Y. D., & Anthony, J. C. (1999). Male-female differences in the earliest stages of drug involvement. *Addiction*, *94*(9), 1413–1419.

van Huijstee, A. N., & Mansvelder, H. D. (2014). Glutamatergic synaptic plasticity in the mesocorticolimbic system in addiction. *Frontiers in Cellular Neuroscience*, *8*, 466. http://doi.org/10.3389/fncel.2014.00466

Vandenberghe, W., Robberecht, W., & Brorson, J. R. (2000). AMPA receptor calcium permeability, GluR2 expression, and selective motoneuron vulnerability. *The Journal of*

Neuroscience : the Official Journal of the Society for Neuroscience, *20*(1), 123–132. http://doi.org/10.1046/j.1471-4159.2000.0740179.x/full

Vanderschuren, L. J. M. J., & Everitt, B. J. (2004). Drug Seeking Becomes Compulsive After Prolonged Cocaine Self-Administration. *Science*, *305*(5686), 1017–1019. http://doi.org/10.1126/science.1098975

Venniro, M., Caprioli, D., & Shaham, Y. (2016). Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence. *Progress in Brain Research*, *224*, 25–52. http://doi.org/10.1016/bs.pbr.2015.08.004

Venniro, M., Zhang, M., Shaham, Y., & Caprioli, D. (2017). Incubation of Methamphetamine but not Heroin Craving After Voluntary Abstinence in Male and Female Rats. *Neuropsychopharmacology*, *42*(5), 1126–1135. http://doi.org/10.1038/npp.2016.287

Vertes, R. P. (2004). Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse*, *51*(1), 32–58. http://doi.org/10.1002/syn.10279

Volkow, N. D., Fowler, J. S., & Wang, G.-J. (2003). The addicted human brain viewed in the light of imaging studies: brain circuits and treatment strategies. *Neuropharmacology*, *47 Suppl 1*, 3–13. http://doi.org/10.1016/j.neuropharm.2004.07.019

Volkow, N. D., Fowler, J. S., Wang, G.-J., Swanson, J. M., & Telang, F. (2007). Dopamine in Drug Abuse and Addiction: Results of Imaging Studies and Treatment Implications. *Archives of Neurology*, *64*(11), 1575–1579. http://doi.org/10.1001/archneur.64.11.1575

Wade, G. N. (1975). Some effects of ovarian hormones on food intake and body weight in female rats. *Journal of Comparative and Physiological Psychology*, *88*(1), 183–193. http://doi.org/10.1037/h0076186

Wade, G. N., & Gray, J. M. (1979). Gonadal effects on food intake and adiposity: a metabolic hypothesis. *Physiology & Behavior*, *22*(3), 583–593.

Walf, A. A., & Frye, C. A. (2005). Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity. *Neuropsychopharmacology*, *30*(7), 1288–1301. http://doi.org/10.1038/sj.npp.1300708

Wang, B., You, Z.-B., & Wise, R. A. (2012). Heroin self-administration experience establishes control of ventral tegmental glutamate release by stress and environmental stimuli. *Neuropsychopharmacology*, *37*(13), 2863–2869. http://doi.org/10.1038/npp.2012.167

Wang, G.-J., Volkow, N. D., Thanos, P. K., & Fowler, J. S. (2009). Imaging of Brain Dopamine Pathways: Implications for Understanding Obesity. *Journal of Addiction Medicine*, *3*(1), 8–18. http://doi.org/10.1097/ADM.0b013e31819a86f7

Warren, M., Frost-Pineda, K., & Gold, M. (2005). Body mass index and marijuana use. *Journal of Addictive Diseases*, *24*(3), 95–100. http://doi.org/10.1300/J069v24n03_08

White, W. L., Boyle, M., & Loveland, D. (2002). Alcoholism/addiction as a chronic disease: From rhetoric to clinical reality. *Alcoholism Treatment Quarterly*. http://doi.org/10.1300/J020v20n03_06

Wise, R. A., & Bozarth, M. A. (1987). A psychomotor stimulant theory of addiction. *Psychological Review*, *94*(4), 469–492.

Wise, R. A., Bin Wang, & You, Z.-B. (2008). Cocaine Serves as a Peripheral Interoceptive Conditioned Stimulus for Central Glutamate and Dopamine Release. *PLoS ONE*, *3*(8), e2846. http://doi.org/10.1371/journal.pone.0002846

Xu, P., Li, M., Bai, Y., Lu, W., Ling, X., & Li, W. (2015). The effects of piracetam on heroin-induced CPP and neuronal apoptosis in rats. *Drug and Alcohol Dependence*, *150*, 141–146. http://doi.org/10.1016/j.drugalcdep.2015.02.026

Yager, L. M., & Robinson, T. E. (2013). A classically conditioned cocaine cue acquires greater control over motivated behavior in rats prone to attribute incentive salience to a food cue. *Psychopharmacology*, *226*(2), 217–228. http://doi.org/10.1007/s00213-012-2890-y

Yager, L. M., Pitchers, K. K., Flagel, S. B., & Robinson, T. E. (2015). Individual variation in the motivational and neurobiological effects of an opioid cue. *Neuropsychopharmacology*, *40*(5), 1269–1277. http://doi.org/10.1038/npp.2014.314

You, Z.-B., Wang, B., Zitzman, D., Azari, S., & Wise, R. A. (2007). A role for conditioned ventral tegmental glutamate release in cocaine seeking. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *27*(39), 10546–10555. http://doi.org/10.1523/JNEUROSCI.2967-07.2007

Yu, J., Zhang, S., Epstein, D. H., Fang, Y., Shi, J., Qin, H., et al. (2007). Gender and stimulus difference in cue-induced responses in abstinent heroin users. *Pharmacology, Biochemistry and Behavior*, *86*(3), 485–492. http://doi.org/10.1016/j.pbb.2007.01.008

Zachariou, V., Bolanos, C. A., Selley, D. E., Theobald, D., Cassidy, M. P., Kelz, M. B., et al. (2006). An essential role for |[Delta]|FosB in the nucleus accumbens in morphine action. *Nature Neuroscience*, *9*(2), 205–211. http://doi.org/10.1038/nn1636

Zacny, J. P., & de Wit, H. (1991). Effects of Food Deprivation on Subjective Effects and Self-Administration of Marijuana in Humans. *Psychological Reports*, *68*(3 suppl), 1263–1274. http://doi.org/10.2466/pr0.1991.68.3c.1263

Zapata, A., Minney, V. L., & Shippenberg, T. S. (2010). Shift from goal-directed to habitual cocaine seeking after prolonged experience in rats. *The Journal of Neuroscience : the Official Journal of the Society for Neuroscience*, *30*(46), 15457–15463. http://doi.org/10.1523/JNEUROSCI.4072-10.2010

Zheng, D., Cabeza de Vaca, S., & Carr, K. D. (2012). Food restriction increases acquisition, persistence and drug prime-induced expression of a cocaine-conditioned place preference in rats. *Pharmacology, Biochemistry and Behavior*, *100*(3), 538–544. http://doi.org/10.1016/j.pbb.2011.10.021

Zheng, D., Cabeza de Vaca, S., Jurkowski, Z., & Carr, K. D. (2015). Nucleus accumbens AMPA receptor involvement in cocaine-conditioned place preference under different dietary conditions in rats. *Psychopharmacology*, *232*(13), 2313–2322. http://doi.org/10.1007/s00213-015-3863-8

Zhou, X., Nonnemaker, J., Sherrill, B., Gilsenan, A. W., Coste, F., & West, R. (2009). Attempts to quit smoking and relapse: factors associated with success or failure from the ATTEMPT cohort study. *Addictive Behaviors*, *34*(4), 365–373. http://doi.org/10.1016/j.addbeh.2008.11.013

Appendix 1: Are the augmenting effects of food restriction on heroin seeking inhibited by a chronic pre-exposure to food restriction?

A1.1 INTRODUCTION

The development of maladaptive dietary habits has often been reported as a consequence of long-term opiate use (Neale, Nettleton, Pickering, & Fischer, 2012). These disturbances in ingestive behaviours are demonstrated by the inadequate and infrequent intake of food (Neale et al., 2012; Noble & McCombie, 1997), which result in chronic malnutrition and the maintenance of low BWs during periods of opiate use (Kaiser, Prendergast, & Editor, 2009; Santolaria-Fernández, 1995). In addition, the intermittent ingestion of foods containing both high sugar and fat content often accompanies the overall reductions in chronic food intake (Nolan & Scagnelli, 2007). However, during periods of drug abstinence, a partial reversal of poor dietary behaviours is marked by increased caloric consumption (Neale et al., 2012). These effects on food consumption are also observed in response to opiate administration in animal models of drug abuse (Levine & Billington, 2004; Marks-Kaufman & Kanarek, 1990). For instance, following acute injections and during prolonged periods of morphine self-administration, rats reduced carbohydrate and protein consumption but increased fat intake (Marks-Kaufman, 1982; Marks-Kaufman & Kanarek, 1990). In our laboratory, we have also reported a maintenance of BW during heroin self-administration, and an increase in both chow consumption and BW within one day of withdrawal from drug taking (D'Cunha et al., 2013).

The relation between disordered eating and drugs of abuse extend beyond the direct effects of drug intake on feeding. This is evidenced by findings suggesting a low comorbidity between obesity and drug abuse (Simon et al., 2006; Warren, Frost-Pineda, & Gold, 2005), and the observed increases in vulnerability to use and relapse to drugs of abuse during periods of dieting (Cheskin et al., 2005; Hall et al., 1992; Krahn et al., 1992). In rodents, augmented drug taking and seeking have also been demonstrated in food-restricted rats (Carr, 2002; Carroll & Meisch, 1981; Keller et al., 2014). Accordingly, we have previously reported increases in heroin-seeking behaviours in chronically food-restricted rats under withdrawal (D'Cunha et al., 2013; Sedki et al., 2015; Sedki, Abbas, Angelis, Martin, et al., 2013a). However, the relation between

self-restricted feeding behaviour observed during drug self-administration and the increased propensity for relapse during drug withdrawal remain unclear. One possibility is that, following repeated pairings of a self-imposed calorically restricted state with drug taking, the restricted state may inherit the properties of a conditioned drug cue through Pavlovian conditioning. As a result, the restricted state can then predict drug availability and increase drug craving. Therefore, re-exposure to a similar state during withdrawal may trigger drug seeking as a consequence of this Pavlovian association. Consistent with this idea, rats that display an aversion to an amphetamine-conditioned environment showed a reversal of this effect when they were food restricted prior to the conditioning period (Cabib, Orsini, Le Moal, & Piazza, 2000).

Furthermore, non-reinforced pre-exposure to a CS can later disrupt or delay the conditioning of that stimulus, a procedure termed latent inhibition (Lubow, 1973). Therefore, if the augmentation of heroin seeking in food-restricted rats results from the previously established association between food restriction and drug taking, then we expect that pre-exposure to restricted feeding conditions should abolish this augmentation. Here, we investigated whether pre-exposure to food restriction may inhibit the augmentation of heroin seeking in food-restricted rats.

A1.2 METHODS

A1.2.1 Subjects

A total of 37 male, Long Evans rats (Charles River, St Constant, Quebec, Canada or New Jersey, USA; 275-300 g) were used. Before surgery, rats were pair-housed for one week in the ACF under 12 h reverse light/dark conditions (lights OFF at 0930 h). After acclimation to the ACF, half of the rats were given restricted access to chow for seven days of pre-exposure. Following a four day recovery from pre-exposure, all rats underwent iIV catheterization, and were then single-housed in plastic shoebox cages and allowed two days of recovery. Next, rats were transferred to operant conditioning chambers for heroin self-administration. Following self-

administration training, rats were returned to the ACF and were single-housed in shoebox cages for the drug-washout and withdrawal periods. Rats were then returned to the operant conditioning chambers for testing. Except for the pre-exposure, withdrawal and testing phases, all rats were allowed 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.

A1.2.2 Surgical Procedures

All rats were implanted with IV silastic catheters (Dow Corning, Midland, MI, USA) under xylazine/ketamine (10.0 + 100.0 mg/kg; ip) anesthesia, as described previously (Sedki, D'Cunha, & Shalev, 2013b). Following surgery, rats were given saline (0.9%) for hydration, penicillin (450 000 IU/rat; s.c.) to prevent infection and the non-steroidal anti-inflammatory ketoprofen (2.0 mg/kg; s.c.; CDMV, QC, CA) to reduce pain. Ketoprofen was administered for 2 additional days following surgery. Catheters were flushed daily throughout the self-administration phase, with heparin/gentamicin (7.5 UI + 12.0 mg/rat) to prevent blockage and infection.

A1.2.3 Apparatus

During self-administration and testing, rats were individually housed in operant conditioning chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 X 29.0 X 25.5 cm) enclosed in a sound attenuating wooden cubicle equipped with a fan (noise rating of 45 dBA; Newark, Canada). The front and back walls of the chambers were built using polycarbonate plexiglass, while the ceiling and side walls were composed of stainless steel. Each chamber was fitted with two retractable levers mounted 9 cm above the floor of the right sidewall. All rats were attached to an infusion pump (3.33 rpm), located above the chamber via a liquid swivel (Lomir Biomedical Inc., Notre-Dame-de-L'île-Perrot, QC, Canada) and Tygon tubing shielded with a metal spring. A red house light was installed at the top center of the left sidewall, while a cue-light/tone complex (Coulbourn Instruments; Sonalert, 2.9 KHz) was located directly above the active (heroin+light+tone-paired) lever.

A1.2.4 Procedure

Pre-exposure (7 days). Before surgery, rats were handled, weighed daily, and allowed unrestricted (Pre-Sated) or restricted (Pre-FDR) access to chow, aimed to reduce their BW to 10% of their baseline weight (pre-exposure day 1). On the first day of pre-exposure, rats in the FDR group had their food removed and were fed ~ 15 g of chow at 1330 h. The amount of food was adjusted daily to ensure the desired weight loss. Rats were allowed four days of recovery before undergoing the IV catheterization surgery.

Self-Administration (10 days). Following recovery from surgery, rats were allowed a 24 h habituation period to the operant conditioning chambers. Next, rats were trained to self-administer heroin in daily, three, 3 h sessions separated by 3 h intervals for 10 days. The first session began within 30 min after the onset of the dark phase with the extension of the levers into the operant conditioning chamber, illumination of a red house light and activation of the cue-light/tone complex for 20 s. Responses on the active (heroin+light+tone-paired) lever, under a fixed interval 20 s schedule of reinforcement (FI-20 s), resulted in the activation of the drug pump (5 s; 0.13 ml/infusion). During the 20 s interval, the house light was turned off, the cue light/tone complex was activated, and active lever responses were recorded but not reinforced. Following each 3 h session, the active lever was retracted whereas the inactive lever was only retracted 1 h prior to the first session on the next day. Alternative (non-paired) lever responses were recorded but had no programmed consequences.

Withdrawal (15 days). Following self-administration training, rats were individually housed in the ACF, and allowed unrestricted access to food and water for one drug-washout day. Rats were then separated into two groups: Sated, or food-restricted (FDR), which were matched according to BW, number of infusions, and active lever responses across the last 5 days of training. On the first day of the withdrawal phase, rats in the FDR group had their food removed and were fed ~ 15 g of chow at 1330 h. The amount of food was adjusted throughout the 14 days of food restriction to maintain the food-restricted rats' BW at ~ 90% of their W1 BW.

Test (1 day). On W14, rats were returned to the operant conditioning chambers and tethered to the metal spring. The heroin-seeking test consisted of one, 1 h session during which active lever responses held the same consequences as in training, excluding the availability of the drug.

A1.2.5 Statistical Analyses

All analyses were conducted using Prism software (Graphpad Software, version 6.0), except for effect sizes and CIs which were calculated using R (The R Project for Statistical Computing, version 3.3.1). The critical threshold for statistically significant results was set at $p \leq 0.05$, and adjusted for multiple comparisons using the Holm-Sidak correction when necessary. All *t*-tests were followed by Cohen's *d* effect size calculations, while all ANOVAs were followed by eta-squared effect size calculations. All CIs reported were calculated around their respective effect sizes.

Active and inactive lever responses during the heroin-seeking test were analyzed using a 2 X 2 between subjects ANOVA with the *pre-feeding condition* (Pre-Sated, Pre-FDR) and the *post-feeding condition* (Post-Sated, Post-FDR) as the between subjects factors.

A1.3 RESULTS

All rats acquired reliable heroin self-administration behaviour, as supported by the mean ± SEM number of infusions (37.43±2.73) and active (113.65±15.30) and alternative (8.92±1.92) lever responses made on the last day of heroin self-administration training. There were no statistically significant differences in any of the reported parameters between the different experimental groups within each experiment.

Three rats were removed from analyses due to sickness ($n = 1$) or experimental error ($n = 2$). Therefore, the final analyses on W14 were conducted on 37 rats across four experimental groups: Pre-Sated-Sated ($n = 10$) and Pre-Sated-FDR ($n = 10$), Pre-FDR-Sated ($n = 8$) and Pre-FDR-FDR ($n = 9$). The average BWs of the Post-Sated rats (484.72±6.93) was statistically

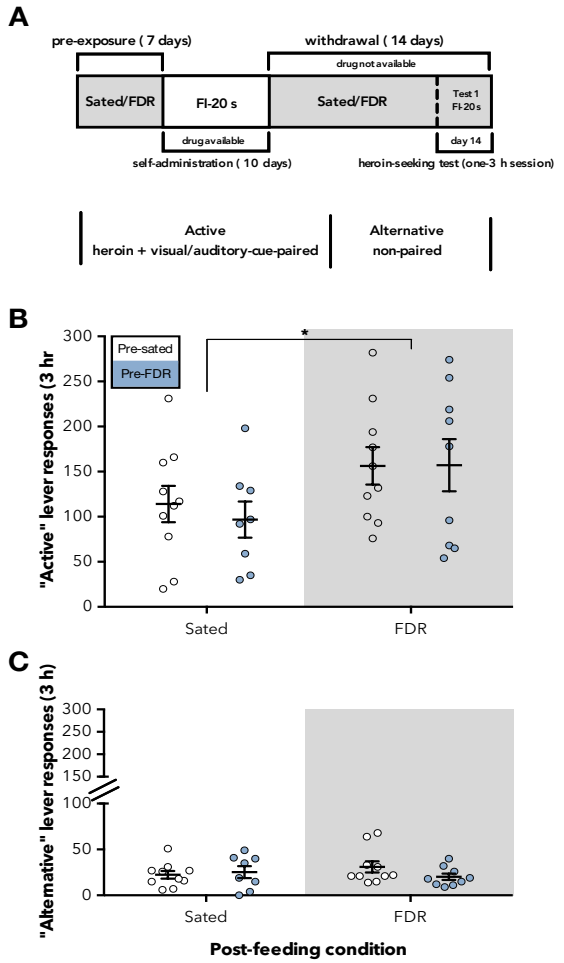

Figure A.1. Pre-exposure to a food-restricted state prior to heroin self-administration and withdrawal does not affect the augmentation of heroin seeking in food-restricted rats. All active and alternative lever responses are depicted as mean (±SEM). Analyses were conducted on 37 rats, in four experimental groups: Pre-sated-sated (n = 10), Pre-sated-FDR (n = 10), Pre-FDR-sated (n = 8) and, Pre-FDR-FDR (n = 9). (**A**) Before self-administration training rats were exposed to 7 days of food-restricted or restricted access to chow. Following heroin self-administration training rats underwent 14 days of withdrawal under sated or FDR conditions. Next, rats underwent one, 3 h heroin-seeking test under extinction conditions. (**B**) The number of active and inactive lever responses during the heroin-seeking test.*****p < .05

significantly greater than that of the Post-FDR (353.74±4.15) rats on W14. This was supported by a statistically significant main effect for the Post-Feeding condition ($F_{(1,33)}$ = 239.90, p < .001, η^2 = 0.88, CI = [0.79, 0.91]), but not the Pre-Feeding condition ($F_{(1,33)}$ = 2.00, p = .167, η^2 = 0.06, CI = [0.00, 0.20]) or the interaction ($F_{(1,33)}$ = 1.04, p = .315, η^2 = 0.0, CI = [0.00, 0.16]) between both conditions.

Rats tested under food-restricted conditions (Post-Feeding condition) exhibited augmented active lever responding, while a prior history of food restriction (Pre-Feeding condition) did not affect lever responding on test day. This is supported by a statistically significant main effect of active lever responses for the post-feeding ($F_{(1,33)}$ = 5.05, p = .031, η^2 = 0.13, CI = [0.006, 0.30]), but not the pre-feeding ($F_{(1,33)}$ = 0.13, p = .718, η^2 = 0.003, CI = [0.00, 0.09]) condition, nor the interaction between both conditions ($F_{(1,33)}$ = 0.16, p = .695, η^2 = 0.004, CI = [0.00, 0.09]). No

statistically significant differences were observed for inactive lever responding (*Pre-feeding* condition: $F_{(1,33)} = 0.56$, $p = .459$, $\eta^2 = 0.02$, $CI = [0.00, 0.13]$; *Post-feeding* condition: $F_{(1,33)} = 0.11$, $p = .743$, $\eta^2 = 0.003$, $CI = [0.00, 0.08]$: *Interaction*: $F_{(1,33)} = 1.75$, $p = .195$, $\eta^2 = 0.05$, $CI = [0.00, 0.19]$; see **Figure A.1**).

A1.4 DISCUSSION

Adaptive behaviours require the ability to adjust learned associations based on previous exposure to the stimuli of interest. In the present study, we pre-exposed subjects to a brief caloric restriction to investigate the learned associations between self-restricted feeding and heroin taking. As previously demonstrated, we observed enhanced drug seeking in chronically restricted, compared to sated rats (Sedki, Abbas, Angelis, Martin, et al., 2013a). Contrary to our hypothesis, a history of food restriction (pre-feeding condition) prior to heroin training was not sufficient to inhibit the augmentation of heroin seeking in chronically food-restricted rats (post-feeding condition) under withdrawal. These data suggest that a learned association between heroin taking and a mild caloric self-restriction does not motivate heroin seeking in food-restricted rats under withdrawal.

It is widely accepted that the rewarding mechanisms which promote addiction-like behaviours also nurture maladaptive feeding behaviours such as overeating and food addiction. Similar to those activated by drug reward, the midbrain DA circuits are believed to regulate the underlying motivation for, and pleasure that arises from, food consumption (Blumenthal & Gold, 2010; G.-J. Wang, Volkow, Thanos, & Fowler, 2009). For example, pre-clinical and clinical studies have revealed a reduction in striatal DA-D2-like receptors in compulsive eaters, and in response to drug abuse (Johnson & Kenny, 2010; Stice, Spoor, Bohon, & Small, 2008; Volkow, Fowler, Wang, Swanson, & Telang, 2007). Furthermore, similar to protracted withdrawal, long-term exposure to a high-fat diet increased levels of ΔFosB within the NAc, a transcription factor believed to enhance the motivation to obtain abused drugs and instigate relapse (Lutter & Nestler, 2009; Nestler, 2005). These similarities extend to drug- and food-associated cues as

well, which are known to activate overlapping circuits such as those within the orbitofrontal cortex, ventral segmental area, substantia nigra, striatum, and lateral hypothalamus (Kenny, 2011a; 2011b). Based on these studies, it has been proposed that abused drugs may command these neural substrates intended to regulate food-related behaviours (Blumenthal & Gold, 2010).

An interpretation of our null findings may be that repeated heroin use encourages a deregulation of the food reward circuits in such a way as to influence the internal sensation of satiety. In that case, the development of a sufficient association between heroin taking and the restricted state may be interrupted or diminished. Interestingly, we found that unrestricted access to chow 2 or 24h before testing prevented the augmentation of heroin seeking in chronically food-restricted rats (D'Cunha et al., 2013). Though rats experienced satiety during the testing phase of that study, it highlights the significance of the internal hunger state on drug-associated behaviours.

An alternative explanation concerns the deregulation of DA transmission within the brain reward system. For instance, rats subjected to a diet designed to promote obesity (i.e, highly palatable, high fat) display reduced baseline mesolimbic DA activity compared to rats fed standard chow of normal BW (Geiger et al., 2009). Furthermore, access to this palatable diet was sufficient to replenish the attenuated DA levels, suggesting that dependence on the palatable diet was necessary to satisfy the desired levels of dopminergic activity. These effects were not observed in animals exposed to standard chow (Geiger et al., 2009). Here, heroin-trained rats self-restricted feeding during drug intake. Therefore, it is possible that food intake, while still pursued for survival, is no longer necessary for dopaminergic homeostasis given this may be achieved through heroin use. While under the influence of the abused drug, we believe that the effects of a mild self-restriction may not be substantial develop a meaningful association between the self-restricted state and heroin intake.

Several methodological considerations may also account for the null findings we have reported. First, the duration of the pre-feeding period was selected in the absence of any parametric analyses. As a result, this period may have been too short, rendering it inadequate in its ability to elicit a blocking effect. Previous research from our laboratory suggested that ten, but not seven days of food restriction was sufficient to reinstate extinguished heroin seeking in rats (Shalev, 2012). Accordingly, the seven day pre-feeding phase was not used to induce drug seeking in this experiment, but rather to provide a primary exposure to the hunger state itself. A second consideration involves the severity of caloric restriction employed (~90% of W1 BW), which may have been insufficient in producing an adequately salient internal state. We argue the relevance of this consideration, as the self-restriction achieved during heroin training is even milder than the experimenter-enforced restriction during the pre-feeding condition. Furthermore, the post-restriction period following training was similar in severity, and was deemed sufficient in its ability to augment heroin seeking during testing.

Next, contextual factors may have been implicated in the lack of differences observed. In our procedure, the pre-exposure phase was conducted in the ACF, while heroin training occurred in operant conditioning chambers in our laboratory. Previous studies measuring latent inhibition have suggested that pre-exposure to the to-be-CS in an environment that differs from the conditioning environment can attenuate or block the latent inhibition effect (Harris, Jones, Bailey, & Westbrook, 2000; Quintero et al., 2011). Thus, we advise that future investigations need to conduct both pre-exposure and conditioning in the same environment to alleviate this concern.

Lastly, the type of imposed food restriction imposed should be considered. During the pre-feeding phase, the restriction was experimenter-controlled, but was then self-imposed throughout the training phase. This difference may be supported by separate circuits, and may also differ qualitatively. While we are unaware of any studies comparing experimenter- versus self-imposed food restriction, several similarities and differences in the underlying circuitry between experimenter-administered and self-administrated drug intake have been reported in

the addiction literature (Robinson et al., 2002). Therefore, we cannot discount the possibility that these distinctive restriction types may have determined our null findings. To address this, future studies should attempt a mild forced restriction during the training period.

Collectively, these findings suggest that the internal state, imposed by caloric self-restriction, is insufficient in both its ability to form a salient association with heroin, and to later promote seeking in food-restricted rats under withdrawal. It is likely that other, more salient cues(e.g., cues presented in association with the drug) inherit the incentive motivational properties that precipitate and augment seeking behaviour. While future studies may consider adjusting both the location of pre-exposure and the type of restriction imposed during training, we posit that studies on other discrete and environmental cues should take precedence.