

The Epigenome:
Possible Mechanisms by which Early Life Stress May Prime
Vulnerability towards Substance Use Disorder

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ABSTRACT

Evidence from the 20th century demonstrated that early life stress (ELS) produces long lasting neuroendocrine and behavioral effects related to an increased vulnerability towards psychiatric illnesses such as major depressive disorder, post-traumatic stress disorder, schizophrenia, and substance use disorder. Substance use disorders (SUDs) are complex neurological and behavioral psychiatric illnesses. The development, maintenance, and relapse of SUDs involve multiple brain systems and are affected by many variables, including socio-economic and genetic factors. Pre-clinical studies demonstrate that ELS affects many of the same systems, such as the reward circuitry and executive function involved with addiction-like behaviors. Previous research has focused on cocaine, ethanol, opiates, and amphetamine, while few studies have investigated ELS and methamphetamine (METH) vulnerability. METH is a highly addictive psychostimulant that when abused, has deleterious effects on the user and society. However, a critical unanswered question remains; how do early life experiences modulate both neural systems and behavior in adulthood? The emerging field of neuroepigenetics provides a potential answer to this question. Methyl CpG binding protein 2 (MeCP2), an epigenetic tag, has emerged as one possible mediator between initial drug use and the transition to addiction. Additionally, there are various neural systems that undergo long lasting epigenetics changes after ELS, such as the response of the hypothalamo-pituitary-adrenal (HPA) axis to stressors. Despite this, little attention has been given to the interactions between ELS, epigenetics, and addiction vulnerability. The studies described herein investigated the effects of ELS on METH self-administration (SA) in adult male rats.

Next, we investigated the effects of ELS and METH SA on MeCP2 expression in the nucleus accumbens and dorsal striatum. Additionally, we investigated the effects of virally-mediated knockdown of MeCP2 expression in the nucleus accumbens core on METH SA, motivation to obtain METH under conditions of increasing behavioral demand, and reinstatement of METH-seeking in rats with and without a history of ELS. The results of these studies provide insights into potential epigenetic mechanisms by which ELS can produce an increased vulnerability to addiction in adulthood. Moreover, these studies shed light on possible novel molecular targets for treating addiction in individuals with a history of ELS.

DEDICATION

This dissertation is dedicated to all people around the world who have been, directly or indirectly, impacted by addiction or the war on drugs.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER	
1 GENERAL INTRODUCTION	1
Early Life Experiences.....	1
Addiction.....	3
Theories of Addiction.....	3
Neuroanatomy and Neurochemistry of Addiction.....	6
Conditioned Learning.....	9
Subjective Effects of Drugs of Abuse.....	10
Rodent Models.....	10
Early Life Stress.....	11
Addiction.....	13
Early Life Stress and Adult Vulnerability Towards Addiction-Like...15	
Methamphetamine.....	16
Epigenetic Mechanisms.....	17
Histone Modifications.....	19
DNA Methylation.....	21
Addiction and MeCP2.....	22
Early Life Stress and Epigenetic Effects.....	25
Glucocorticoid Receptor (Nr3c1).....	26

CHAPTER	Page
Arginine Vasopressin.....	28
Brain Derived Neurotrophic Factor.....	28
5-HT Transporter (Slc6a4).....	29
GABA and Glutamate.....	30
Epigenetic Machinery.....	31
Summary.....	31
2 THE EFFECTS OF MATERNAL SEPARATION ON METHAMPHETAMINE SELF-ADMINISTRATION, EXTINCTION, AND CUE INDUCED REINSTATEMENT	33
Abbreviated Methods.....	37
Animals.....	37
Behavioral Testing.....	37
Tissue Preparatin, Immunohistochemistry, and Analysis.....	38
Results.....	38
Litter Effects.....	38
METH Self-Administration.....	38
Extinction.....	39
Cue-Induced Reinstatement.....	39
MeCP2 Immunoreactivity.....	39
Discussion.....	40

CHAPTER	Page
3	EARLY LIFE STRESS INFLUENCES METHAMPHETAMINE INTAKE BEHAVIOR THROUGH MODIFICATION OF MECP2 EXPRESSION IN THE NUCLEUS ACCUMBENS CORE 46
	Abbreviated Methods.....50
	Study 1.....50
	Animals.....50
	Behavioral Testing.....50
	Study 2.....51
	Animals.....51
	Behavioral Testing.....51
	Results.....52
	Study 1.....52
	Study 2.....54
	Discussion.....56
4	THE EFFECTS OF MATERNAL SEPARATION AND ADULT CHRONIC VARIABLE STRESS ON METHAMPHETAMINE SELF- ADMINISTRATION, EXTINCTION, AND CUE INDUCED REINSTATEMENT 62
	Abbreviated Methods.....63
	Results64
	Discussion.....65

CHAPTER	Page
5 THE EFFECT OF LITTER SEPARATION ON DAM [♀] 'S CONDITIONED PLACE PREFERENCE TO METHAMPHETAMINE	67
Abbreviated Methods.....	71
Animals, Litter Separation, and Stress Procedures.....	71
Methamphetamine Conditioned Place Preference.....	71
Corticosterone Assay.....	72
Results.....	72
Estrous Cycle.....	72
CPP Score.....	73
Locomotor Activity.....	73
Plasma Corticosterone Levels.....	73
Discussion.....	74
6 GENERAL DISCUSSION	78
Early Life Stress, MeCP2 Expression, and METH Addictive-Like.....	78
Early Life Stress, Adult Chronic Stress, and METH Intake Behavior...	79
Postpartum Stress and Conditioned Reward to METH	80
Final Conclusions.....	81
REFERENCES.....	84
 APPENDIX	
A ANIMALS AND MATERNAL SEPARATION PROCEDURES	111
B BILATERAL INTRACEREBRAL VIRUS INJECTION	114
C CHRONIC VARIABLE STRESS EXPOSURE	116

APPENDIX	Page
D CONDITIONED PLACE PREFERENCE.....	118
E IMMUNOBLOT ANALYSIS.....	120
F IMMUNOHISTOCHEMISTRY.....	122
G JUGULAR VEIN CATHETER SURGICAL PROCEDURES.....	125
H RESTRAINT STRESS AND BLOOD COLLECTION.....	127
I ROTATIONAL MOTOR ACTIVITY.....	129
J SELF-ADMINISTRATION APPARATUS.....	131
K SELF-ADMINISTRATION, EXTINCTION, PROGRESSIVE RATIO, AND CUE- INDUCED AND DRUG-INDUCED REINSTATEMENT.....	133
L SH-RNA VIRUS DESIGN.....	136
M TRANSCARDIAL PERFUSION.....	138

LIST OF TABLES

Table		Page
1.	Mean \pm SEM Lever Presses Across 15 Sessions of METH SA.....	140
2.	Example Chronic Variable Stress Procedure.....	141
3.	Effect of Acute Restraint Stress on Plasma CORT Levels.....	142

LIST OF FIGURES

Figure		Page
1.	Average METH Infusions Over 15 Days.....	143
2.	Total METH Infusions	144
3.	Average Active Responses During Extinction Training	145
4.	Cue-Induced Reinstatement Testing	146
5.	MeCP2 Immunoreactivity in the NAc core	147
6.	Average Active Responses During Extinction Training	148
7.	Representative Photomicrograph of NAcc MeCP2 Immunolabeling	149
8.	Average METH Infusions Over 11 Days.....	150
9.	Average Active Responses Over 11 Days	151
10.	MeCP2 Immunoreactivity in the NAc Core	152
11.	Representative Photomicrograph of NAcc MeCP2 Immunolabeling	153
12.	MeCP2 Immunoreactivity in the NAc Shell	154
13.	MeCP2 Immunoreactivity in the Medial Dorsal Striatum.....	155
14.	MeCP2 Immunoreactivity in the Lateral Dorsal Striatum.....	156
15.	Representative Bands of MeCP2 and GAPDH Protein Expression.....	157
16.	Representative Photomicrograph of the Viral Surgery Placement.....	158
17.	Average METH Infusions Over 10 Days.....	159
18.	Active Responses Breakpoint for METH Progressive Ratio	160
19.	Infusions Breakpoint for METH Progressive Ratio	161
20.	METH Primed Reinstatement	162
21.	Average Sacc Reinforcers Over 19 Days.....	163

Figure	Page
22. Active Responses Breakpoint for Sacc Progressive Ratio	164
23. Earned Reinforcers Breakpoint for Sacc Progressive Ratio	165
24. Cue-Primed Reinstatement for Sacc.....	166
25. Rotational Motor Activity.....	167
26. Average METH Infusions Over 15 Days.....	168
27. Average Active Responses for METH.....	169
28. Average Active Responses During Extinction Training	170
29. Cue-Primed Reinstatement	171
30. METH CPP Score	172
31. Saline CPP Score	173
32. Locomotor Activity.....	174

CHAPTER 1

GENERAL INTRODUCTION

Early Life Experiences

Visionary psychologists of the 20th century produced findings that prompted a movement of research investigating how early life experiences help shape an individual's development into maturation. At the turn of the 20th century, pioneering psychologist Elmer Gates (1904) referred to early life experiences mediating brain development as a “process of brain-building”. A few decades later, Donald Hebb (1947) demonstrated that cognitive performance in rats was enhanced by different, particularly more enriched, rearing environments. The next decade produced literature from Victor Denenberg's laboratory demonstrating a lasting neuroendocrine effect of early life handling in rodents (Levine et al., 1967). Around the same time, Harry Harlow demonstrated that decreased quality of maternal care had long lasting detrimental effects on behavior in non-human primates (Harlow et al., 1965). Michael Meaney (1985) elaborated on Denenberg's findings by establishing a relationship between early life handling experience and adult hippocampal glucocorticoid receptor expression. Meaney expanded upon this work in a seminal paper (Plotsky & Meaney, 1993) in which a group of animals with extended maternal separation was added to the paradigm. Soon after, it was discerned that maternal care, specifically licking, grooming, and arch back nursing (LG or LG-abn), was driving the differences observed in handling groups (Liu et al., 1997). The majority of rodent paradigms modeling ELS used today are modified versions of paradigms based on this collection of work.

Exposure to ELS such as traumatic events, abuse, and neglect is highly related to one's health in adulthood. Multiple variables within the home such as parenting style and the quality of the parent/child relationship can affect a child's physical and intellectual development (Anda et al., 2006; Bremne & Vermetten, 2001; Lovallo et al., 2012; Schimmenti & Bifulco, 2013). For example, in individuals with a history of ELS, there is an increased risk for the development of physical illnesses such as obesity, diabetes, seizures, and cardiovascular diseases (Gluckman et al., 2008; Gunstad et al., 2006; Huang, 2014). Additionally, aggressive and suicidal behaviors are strongly associated with ELS (McEwen, 2003). Furthermore, ELS is a risk factor for the development of various psychiatric illnesses such as personality disorders, major depression, post-traumatic stress disorder, schizophrenia, and SUDs (De Bellis, 2002; Pechtel & Pizzagalli, 2011; Teicher et al., 2003).

ELS is a prevalent phenomenon with highly detrimental costs to societies worldwide (De Bellis & Zisk, 2014) such as the increased propensity towards SUDs. Clinical research has shown ELS, particularly childhood abuse and neglect, is a reliable risk factor that influences adult drug abuse (Anda et al., 2006; Messina et al., 2008). Childhood abuse or neglect is highly prevalent with ~1.5 million cases reported in 2010 (Child Maltreatment 2010, US Department of Health and Human Services) and there is substantial evidence that ELS produces long-lasting changes in the brain, including regions that mediate reward-seeking and executive control, which may ultimately predispose the individual to increased propensity toward illicit drug use and addiction (Matthews et al., 2001; Meaney et al., 2002). To understand the relationship between

ELS and addiction vulnerability, we first must briefly discuss the etiology and pathology of addiction.

Addiction

Drug addiction, or more formally SUD, is a serious and chronic disease wherein the afflicted individual has difficulty limiting drug intake, exhibits high motivation to take one or more drugs, continues using the drug despite negative consequences, and experiences negative emotional states when the drug is withheld, leading to frequent relapse (American Psychiatric Association, 2013). In the United States, the 2010 prevalence rates (current and past month use, in persons 12 years or older) for illicit drug use (including marijuana, cocaine, and heroin) reached 22.6 million (8.9%) (SAMHSA, 2010). The 2010 prevalence rate for alcohol use was 131.3 million (51.8%), binge alcohol use was 58.6 million (23.1%) while tobacco use was 69.6 million (27.4%) (SAMHSA, 2010). The estimated number of persons 12 years or older classified with substance dependence (including illicit drugs and alcohol) in 2010 was 22.1 million, representing 8.7% of the population (SAMHSA, 2010). Furthermore, 20.5 million Americans were classified as needing treatment for an illicit drug or alcohol use problem (SAMHSA, 2010). These astonishingly high numbers clearly indicate that drug addiction and substance dependence remain significant problems in the United States.

Theories of addiction.

The transition from drug use to drug dependence is impacted by a number of factors, including genetics, environmental influences (such as stress), neurochemical, and neuroanatomical modifications in the brain that result from repeated drug use (Li et al., 2011; Sinha, 2008). Several theoretical frameworks have been put forward in order to

understand the complexities of this transition. Initial drug use can be attributed to the ability of the drug to act as a reward (i.e. induce a pleasurable emotional state) or positive reinforcer (a stimulus that causes the behavior preceding its obtainment to increase), both of which can lead to repeated drug use and dependence (Wise, 1980; Stolerman, 1992). A great deal of research has focused on the molecular mechanisms underlying the initial rewarding or reinforcing effect of drugs of abuse. However, more recent research on long-term neuroanatomical and molecular changes in the brain that result from chronic drug use have revealed drug addiction to be highly complex and involve brain systems beyond the reward circuitry.

Several parallel and intersecting theories of drug addiction include shifts in behavior that are supported by various changes in the underlying neurocircuitry of addiction. Robinson and Berridge hypothesized the incentive sensitization theory, whereby repeated drug use produces alterations in brain reward and associative learning systems, so that the drug user becomes increasingly sensitive to both the drug and drug associated cues, resulting in pathological drug seeking or wanting (Robinson & Berridge, 1993; Berridge & Robinson, 1995). Koob and colleagues have postulated the allostasis theory, which asserts that repeated drug use and dependence are a result of the decreased function of the brain's reward system coupled with an increase in the engagement of the brain's anti-reward or stress circuitries (Koob & Le Moal, 1997; Koob & Le Moal, 2008). These two opposing neurocircuitries are hypothesized to interact in a cyclical manner that ultimately manifest in addiction (Koob & Le Moal, 2008). Everitt and colleagues have theorized that drug addiction is the result of transitions from initial, voluntary drug use governed by stimulus-response relationships towards habitual and eventually compulsive

drug use, which result from a hypothesized switch in the relative engagement of the neurocircuitries underlying these behaviors (Everitt & Robbins, 2005). It has also been suggested that addiction is a disorder of learning and memory, characterized by the ability of drugs to “hijack” normal learning and memory processes that regulate reward seeking (Hyman, 2005).

Another common theory of addiction is cortical 'hypofrontality', specifically referring to reduced baseline activity of the prefrontal cortex which results in an inability to exert executive control over self-regulation. In contrast, many regions become hypersensitive to drugs of abuse as well as drug-associated cues. Several regions of frontal cortex, including the prefrontal cortex, anterior cingulate cortex and orbitofrontal cortex have glutamatergic projections to the nucleus accumbens (NAc) and ventral tegmental area (VTA), and control various aspects of executive function such as working memory, attention, and behavioral inhibition (Nestler, 2005).

These theories all agree that drug addiction is characterized by a progression or shift from initial stages where the drug user is in control of drug intake, to the end stages where the user has lost control over drug intake. Furthermore, this continuum of behavioral adaptation is proposed to be mediated by drug-induced alterations in the neurocircuitry underlying reward, learning and memory and decision-making. Despite these recent advances in our understanding of the addicted brain, the majority of Food and Drug Administration (FDA) approved treatments for drug addiction (few as there are) aim to intervene at the level of the acute rewarding effects of drugs of abuse. Moreover, there are currently no FDA-approved medications for the treatment of addictive disorders involving psychostimulants such as METH.

Neuroanatomy and neurochemistry of addiction.

The brain reward circuitry underlies motivated behavior for both natural rewards (e.g., food, drink and sex) as well as drug rewards. Some of the key brain regions of the reward circuitry include the VTA, NAc, prefrontal cortex (PFC), dorsal striatum (DS) and amygdala (AMG). The rewarding effects of drugs and the progression from initial drug use to dependence are highly dependent on complex interactions between these regions. Dopamine (DA) has historically been thought of as the primary neurotransmitter mediating reward. However, many drugs of abuse also increase extracellular levels of glutamate (Glu), norepinephrine (NE), serotonin (5-HT) and other neurotransmitters within this neurocircuitry, which also play a key role in the long-term molecular and behavioral features of addiction (Kalivas, 2009; Hyman & Malenka, 2001).

Reward.

Ventral tegmental area.

The brain reward circuit functionally begins with the VTA, which contains a cluster of dopaminergic neurons with efferent projections to the NAc in addition to the PFC, basal ganglia and AMG. Many natural rewards and drugs of abuse activate (either directly or indirectly) dopaminergic neurons of the VTA to release dopamine (DA) in these forebrain regions (Robinson & Berridge, 1993; McClure et al., 2003; Berridge & Robinson, 1998; Di Chiara & Imperato, 1988). Drugs of abuse also induce DA release at the level of the terminal fields in the NAc, PFC and AMG (Koob & Volkow, 2010). The release of DA in the NAc is crucial for the rewarding effects of drugs of abuse. In addition, drug-induced DA release in the NAc and other brain regions of the reward circuitry is modulated by other neurotransmitter systems including GABA, Glu,

endogenous opioids, 5-HT and NE (Doherty & Gratton, 1997; Kalivas, 2009; Koob, 1992; Mermelstein & Becker, 1995; Mitchel & Gratton, 1991). The VTA is modulated by glutamatergic inputs from the PFC and AMG in addition to a subpopulation of inhibitory GABAergic interneurons within the VTA (Johnson & North, 1992). Repeated drug use or exposure results in numerous cellular and molecular changes that contribute to reward associated learning and the development of addiction (Jay, 2003).

Nucleus accumbens.

All drugs of abuse increase extracellular levels of DA in the NAc (Di Chiara & Imperato, 1988). The NAc, which is contained within the ventral striatum, is composed primarily of GABAergic medium spiny neurons. The NAc can be functionally divided into two distinct subregions, the core and shell, which respond to rewarding stimuli differently and are thought to mediate distinct aspects of addictive processes. The release of DA in the NAc shell is believed to contribute to the initial rewarding and reinforcing effects of drugs and the assignment of salience to rewarding stimuli (Sellings & Clarke, 2003; Bassareo & Di Chiara, 1999; Everitt et al., 2008), whereas DA release in the NAc core is thought to be more associated with the expression of learned behaviors in response to rewarding stimuli, such as drug-seeking (Kelley, 2004; Di Ciano & Everitt, 2001).

Executive function.

The PFC is a key regulator of cognitive and emotional processes, and contains many functionally distinct subregions. The dorsolateral PFC, orbitofrontal cortex and anterior cingulate cortex are involved the assignment of saliency, decision-making, inhibition of inappropriate behaviors, behavioral and cognitive flexibility, and the

regulation of emotion (Volkow et al., 2011; Breiter et al., 1997). These regions of the PFC have dense populations of glutamatergic cell bodies with efferent pathways that synapse onto the same cell populations in the NAc that receive dopaminergic afferents from the VTA (Sesack & Pickel, 1992). In addition, glutamatergic efferents from the PFC also project to the AMG and VTA (Kalivas & Volkow, 2005). Thus, the PFC is situated to modulate the rewarding effects of drugs of abuse, drug-seeking behavior, as well as relapse to drug taking. Furthermore, the PFC is itself modulated by DA input from the VTA and other input from subcortical structures such as the striatum (Taber et al., 1995; Karreman & Moghaddam, 1996; Brake et al., 2000; Feil et al., 2010).

Action and inhibition.

Basal ganglia circuitry.

While the PFC coordinates the flexibility with which an organism adapts its goal-directed behavior, it is modulated by inputs from other cortical and subcortical regions (Blasi et al., 2006). The basal ganglia are a complex subcortical network of nuclei, consisting of the ventral (Nac) and DS (a.k.a. caudate putamen), the globus pallidus (internal and external segments), substantia nigra, and subthalamic nucleus (Kopell & Greenberg, 2008). The output of the basal ganglia is predominantly via the thalamus which then projects back to the prefrontal cortex, to form cortico-striatal-thalamo-cortical (CSTC) loops. Three CSTC loops are proposed to modulate executive function, action selection and behavioral inhibition. In the dorsolateral prefrontal circuit, the basal ganglia primarily modulate the identification and selection of goals, including rewards (Feil et al., 2010). The orbitofrontal circuit modulates decision-making and impulsivity and the anterior cingulate circuit modulates the assessment of consequences (Feil et al.,

2010). These circuits are modulated by dopaminergic inputs from the VTA to the ventral striatum (NAc) and PFC to ultimately guide behaviors relevant to addiction including persistent drug seeking, impulsive and compulsive drug taking, and the continued drug use despite negative consequences (Feil et al., 2010; Kopell & Greenberg, 2008; Sesack et al., 2003).

Dorsal striatum.

The DS (caudate putamen) is part of the basal ganglia. The DS is further associated with transitions from goal-directed to habitual drug use, due in part to its role in stimulus-response learning (Everitt et al., 2008; Yin et al., 2004). As described above, the initial rewarding and reinforcing effects of drugs of abuse are mediated by increases in extracellular DA in the ventral striatum (NAc shell and after continued drug use, the NAc core) (Di Chiara et al., 2004; Ito et al., 2004). After prolonged drug use, drug associated cues produce increases in extracellular DA levels the DS and less so in the ventral striatum (Ito et al., 2002). This lends to the notion that a shift in the relative engagement from the ventral to the DS underlies the progression from initial, voluntary drug use to habitual and compulsive drug use (Everitt et al., 2008).

Conditioned learning.

The AMG plays a prominent role in the conditioned learning, where strong associations between environmental stimuli and the rewarding of effects of drug use are made. Glutamatergic efferents from the AMG to the PFC and NAc mediate behavioral responses to these stimuli (Cardinal et al., 2002). The AMG is involved in two circuits that mediate stimulus-reward associations. The AMG-mediodorsal thalamus-PFC subcircuit has been implicated in the formation of stimulus-reward associations (Gaffan

& Murray, 1990). The second subcircuit between the AMG, VTA and NAc is more closely linked with the unconditioned rewarding effects of natural and drug rewards, in addition to the expression of stimulus-induced drug-seeking (Everitt et al., 1991). Within this circuit, the basolateral AMG is critical for cue-induced relapse (Everitt et al., 1991; Everitt & Wolf, 2002). In addition, the central nucleus of the AMG is a particularly key neural substrate underlying stress-induced relapse (Shalev et al., 2002; Shaham et al., 2003).

Subjective effects of drugs of abuse.

Some of the most frequently abused substances include alcohol, nicotine, cannabis (marijuana, hashish), psychostimulants (e.g., cocaine and amphetamines) and opioids (e.g. morphine, heroin, and prescription opioids). Drugs of abuse produce pleasurable emotional states and/or relieve negative emotional states, but the specific subjective effects differ by drug type. For example, alcohol, opiates, and cannabinoids produce disinhibition, analgesia, relaxation, and feelings of euphoria in addition to motor and memory impairments (National Institute on Drug Abuse, 2010a; 2011). On the other hand, stimulants such as cocaine and amphetamines are associated with euphoria, increased energy, wakefulness, increased activity, and decreased appetite (National Institute on Drug Abuse, 2006; 2010b). Nicotine produces mild stimulant effects, without euphoria, and can also enhance cognitive processes (Rusted et al., 2009; Holmes et al., 2008; Lawrence et al., 2002; Green et al., 2005).

Rodent Models

Addiction has a variety of detrimental health, social, and economic consequences, therefore, it is important to understand the neural underpinnings of the

relationship between ELS and the increased vulnerability towards addiction of the victims. Elucidating the neural mechanisms of this relationship is of great value in the pursuit of possible treatment and intervention. One way to achieve this is investigations using animal models. In the rat, it is well known that ELS has profound impacts because of plastic neurological development during the first two weeks of life. As a part of normal development, rat pups undergo a stress hypo-responsive period (SHRP) during the first two weeks of life, whereby the HPA axis is relatively unresponsive (Levine, 1994). There may be a similar hyposensitive period in human infants (Gunnar & Donzella, 2002). Preclinical studies show that when maternal care is disrupted for long periods, stress hormones are abnormally elevated in infants during the SHRP. Exposing the developing brain to stress and stress hormones during the SHRP period causes a multitude of neurobiological changes. For example, rats exposed to stress during the SHRP demonstrate alterations in monoaminergic systems, the primary excitatory and inhibitory neurotransmitter systems, as well as the neuroendocrine stress responses in adulthood (Meaney, 2001). Therefore, rodent paradigms that model both ELS and addiction like behaviors have been developed to further study these phenomena. Below, we will briefly discuss prominent rodent paradigms that model both ELS and addiction.

Early Life Stress.

There is an abundant amount of literature investigating the lasting neurobiological and behavioral effects of ELS in rodents. Within this body of literature, multiple paradigms exist including maternal separation, maternal deprivation, naturally occurring low levels of maternal behavior, and brief exposure to an abusive female. Within the body of literature investigating the effects of ELS and vulnerability towards addiction,

there are two primary rodent models of ELS. First, in the maternal separation/deprivation paradigms (in which pups are removed from maternal care during the first 1-2 weeks of life) modeled after Plotsky and Meaney's (1993) original paradigm, there is an unfortunate amount of variability in various parameters, ranging from manipulation of litter composition, cross-fostering, number of days of separation, time and length of separation, separation environment, day of weaning, and housing conditions during adolescence. Furthermore, the appropriate use of a control group has also received considerable debate (Matthews et al., 2001; Matthews et al., 1999). In general, the adult phenotype of pups exposed to ELS consists of a hyperactive HPA axis, increased anxiety-like and depressive behaviors, increased drug intake, a compromised immune system, and alterations in monoaminergic systems (Ladd et al., 2000; Lippmann et al., 2007; Meaney, 2001).

Second, Meaney and colleagues postulated that the change in adult offspring phenotype after maternal separation was due to a change in maternal behavior rather than the actual physical separation (Liu et al., 1997). This spurred an investigation into the natural variations in maternal care, which ultimately led to the development of another paradigm to investigate the effects of early life experiences. In this paradigm, pups are not physically separated from their mothers, but instead maternal care is coded daily in order to define which litters receive high maternal care in the form of licking, grooming, and arch back nursing (designated "H-LG" for high licking and grooming) or low maternal care (designated "L-LG" for low licking and grooming) (Liu et al., 1997). Adult offspring who received L-LG maternal behavior develop similar phenotypes as pups exposed to separation/deprivation procedures. The consistent manipulation across these

paradigms is the disruption of maternal care during the SHRP, followed by the evaluation of effects in adulthood. For descriptions of other less frequently used ELS rodent paradigms, see Lutz and Turecki (2013) for a review.

Addiction.

While human research can elucidate many factors that contribute to the characteristics and cycle of addiction, animal research is necessary to fully examine the neurobiological consequences of repeated drug use. Additionally, animal models aid in the investigation of neuropharmacological actions of drugs of abuse and efficacy of pharmacological treatments for addiction. Furthermore, utilizing animal models of addiction allows researchers to investigate the addictive potential of drugs without administering them to humans. Common examples of the most frequently used animal models of addiction include intracranial self-stimulation (ICSS), conditioned place preference (CPP), behavioral sensitization, and self-administration (SA) paradigms with subsequent extinction training and reinstatement (Balster, 1991). Here we will briefly review the basic design, benefits, and limitations of CPP, SA, extinction training, and reinstatement procedures.

CPP is based on principles of classical (associative) conditioning that assert that if a drug has rewarding or reinforcing properties, then an animal will prefer a context with which the drug has been repeatedly paired. Researchers use a two- or three-chambered apparatus which differs from one side to the other on many variables such as color, texture, and lighting. The animals are then repeatedly conditioned with saline on one side and a drug on the other over a period of 1-2 weeks. After conditioning, the animal is allowed to freely explore either side of the apparatus. CPP is considered to have occurred

if the animal spends more time in the drug-paired context as compared to the saline-paired context. Multiple drugs of abuse can induce a CPP including opiates, nicotine, cocaine, and METH (Feltenstein & See, 2008; Hensleigh & Pritchard, 2014). CPP is a useful paradigm as it is a relatively fast and inexpensive procedure, requires no animal surgery, and little behavioral training. Some limitations, however, include variations in how the drug is introduced to the animal (i.e., intraperitoneal vs. subcutaneous injections, neither of which are voluntarily administered by the experimental subject), number of conditioning sessions, and the lack of established drug dependence. Additionally, dose-effects curves are difficult to assess (Feltenstein & See, 2008).

Arguably the best rodent model of addiction to date is the SA paradigm. In this paradigm, rodents are trained to complete an operant response, such as a lever press or a nose-poke into a specified aperture, in order to receive a drug reinforcer. For over half a century, researchers have found that rodents will self-administer opiates, cannabinoids, alcohol, nicotine, amphetamine, METH, and cocaine to a similar extent as humans, although some drugs are more readily self-administered than others (Feltenstein & See, 2008). SA paradigms can differ by the route of administration. For example, rodents and primates can be trained to self-administer drugs orally, intracranially, or intravenously by a surgically implanted catheter. This paradigm also offers the advantage of providing evidence for the abuse potential of novel drugs. Additionally, subjecting the animal to extinction training (i.e., withholding the drug reinforcer) provides a model for drug seeking behavior and drug-related inhibitory learning in humans. Furthermore, reinstatement after extinction can be induced by three primary modalities: acute drug exposure (drug prime), stress, or exposure to drug associated cues, all of which model

relapse behavior in humans. Though, theoretical limitations of the reinstatement paradigm exist, such that no drug is obtained from the seeking behavior, therefore the rat does not actually experience relapse.

Early Life Stress and Adult Vulnerability Towards Addiction-Like Behaviors

The maternal separation (MS) paradigm is commonly used to investigate the influences of early life events on addictive behaviors. Many neural systems implicated in drug addiction are influenced by MS, such as the HPA axis (Plotsky & Meaney, 1993), endocannabinoid system (Romano-López et al., 2012), monoaminergic systems (Dimatelis et al., 2012a; Matthews et al., 2001; Ploj et al., 2003), and neurotrophic factors such as brain derived neurotrophic factor (BDNF) (Bolaños & Nestler, 2004; Lippmann et al., 2007). In general, adult pups previously exposed to MS for several hours per day exhibit depression-like symptoms, anxiety-like behavior, and an exaggerated neuroendocrine responses to stress (Huot et al., 2001). MS has been reported to also alter the reinforcing effects of cocaine, ethanol, amphetamine, and morphine (Der-Avakian & Markou, 2010; Huot et al., 2001; Moffett et al., 2006; Vazquez et al., 2005). However, only a few reports have been published on the effects of MS on adult METH vulnerability. In one study, MS failed to produce a significant increase in METH CPP in adolescent rodents (Faure et al., 2009; Hensleigh & Pritchard, 2014), while another study demonstrated that MS attenuated METH CPP in adolescent rats (Dimatelis et al., 2012a). MS has also been shown to produce a sex- and dose-dependent increase in locomotor and stereotypy responses to METH in adolescent rats (Pritchard et al., 2012). Recently, we published the first report on the effect of MS on adult intravenous METH self-administration (SA), extinction, and reinstatement (see Chapter 2 and Lewis et al., 2013).

In order to understand the need for continued research concerning the interactions between ELS and METH addiction, we will briefly discuss this highly addictive substance in more detail.

Methamphetamine

METH is an extremely potent and highly addictive neurotoxic drug in the phenylethylamine class of psychostimulants (Vearrier et al., 2012; Xie & Miller, 2009). METH has a range of potential medical uses from attention deficit hyperactivity disorder treatment to narcolepsy, asthma, and obesity treatments (Marshall & O'Dell, 2012). METH is an N-methylated form of amphetamine, which increases lipid solubility thus allowing for a more rapid crossing of the blood-brain barrier. METH exerts its central nervous system effects through interactions with plasma membrane monoamine reuptake transporters and intracellular vesicular monoamine transporters (VMAT) located in presynaptic terminals, acting as a pseudo-substrate for DA, NE and 5-HT transporters (Sulzer, 2011). METH is transported into the presynaptic terminal where it also is transported into synaptic vesicles by VMATs, where it causes a redistribution of vesicular monoamines to the cytoplasm. This results in a reversal of the directionality of plasma membrane transporters and a subsequent flow of monoamines out of the presynaptic terminal into the extracellular space (Ciccarone, 2011). While METH increases NE and 5-HT levels in the NAc, increases in extracellular DA plays a more prominent role in at least the initial rewarding and reinforcing properties of METH (Sulzer, 2011).

METH abuse has many detrimental consequences for the individual and for society as a whole. For the individual, chronic abuse has negative neuropsychological and

psychiatric effects, as well as modifying the healthy brain's functional and structural reward and learning neurocircuitry (Bernacer et al., 2013; Darke et al., 2008; Taylor et al., 2013). METH abuse has been identified as both a strong risk-factor for violence and high-risk sexual behaviors. In one study of a population between the ages of 18 and 25, 34.9% of respondents self-reported violent behavior while under the influence of METH, such as domestic violence, gang-related violence, and random acts of violence (Sommers et al., 2006). Individuals on METH often engage in unprotected vaginal and anal sex and also have unprotected sex with multiple partners (Springer et al., 2007). Acute METH use has many effects such as dilated pupils, dry mouth, sweating, numbness, and increased heart rate, blood pressure, and body temperature. Chronic METH use has a wide range of potential negative health consequences such as damage to oral health, behavioral disturbances, increased risk of stroke and heart attack, and liver and kidney damage. It is apparent that chronic METH use has a multitude of deleterious effects on the users, which in turn has a negative outcome on society.

Epigenetic Mechanisms

Exposure to childhood abuse and household dysfunction has been related to an earlier onset of METH use in both men and women (Messina et al., 2008), and while preclinical work demonstrates that the deleterious effects of ELS on neurodevelopment are abundant, many relevant questions have gone unanswered for decades. How does an experience early in life effectively change the development of a complex system such as the brain? Why do some but not all people who experience ELS develop psychiatric illnesses in adulthood? Recent evidence has suggested epigenetics as a possible mechanism to answer these and many other questions. One of the leaders of the field,

David Sweatt (2013), reviewed the striking increase in epigenetics research over the last 15 years. Within this influx of research is an impressive body of literature demonstrating that DNA methylation and histone modifications in neuronal cells are modulated by experiences, both in early life and adulthood. This new field, referred to as ‘neuroepigenetics’ (Sweatt, 2013) or ‘neurobehavioral epigenetics (Lutz & Turecki, 2013), indicates that a myriad of external factors including nutritional status, education and socioeconomic status (SES), exposure to environmental toxins and drugs of abuse, withdrawal from chronic drug intake, and stress can all influence epigenetic regulators of gene expression. Gene expression in turn influences behavior, phenotypes, and vulnerability to various psychiatric illnesses. The concept that our adult genetic profile is shaped partially based on our earliest experiences is an innovative paradigm shift from traditional views of neurodevelopment (Sweatt, 2013). It also addresses the long-standing debate of nature versus nurture in the field of psychology; it is no longer nature versus nurture, but now it is undeniably nature and nurture that determine the sum of the complex individual.

The word “epigenetics” comes from the Greek root ‘epi’ meaning ‘above’, ‘over’, or ‘in addition to’. This refers to the regulation of gene expression outside of genomic information in the DNA nucleotide sequence (Biliński et al., 2012). In 1942, the developmental biologist Conrad H. Waddington introduced this term and the ‘epigenetic landscape’ to explain the process by which multicellular organisms develop different phenotypes independent of their identical genome (Waddington, 1942). Through activation and repression of specific genes, cells differentiate into distinct phenotypes such that a liver cell is distinct from a muscle cell and so forth. The epigenetic cellular

profile is then inherited by the daughter cells and is maintained through cellular division, thus liver cells remain liver cells and muscle cells remain muscle cells for the lifespan of the organism. This traditional definition of epigenetics included ‘heritability’ as a qualifier. In more recent decades it has become apparent that post-mitotic cells, such as neurons, go through rapid dynamic processes that modulate gene expression (Narayan & Dragunow, 2010; Renthal & Nestler, 2008; Roth, 2012; Tsankova et al., 2007). Hence, the term ‘epigenetics’ and the debate over its accurate definition spans many disciplines (Ho & Burggren, 2010).

There are numerous epigenetic mechanisms, but the most commonly studied are post-translational modifications to histones and DNA methylation. Other mechanisms such as non-coding RNAs (e.g., microRNAs and others), prion proteins, and histone remodeling, though emerging as relevant processes, are beyond the scope of this review. Briefly described here are the mechanisms underlying post-translational histone modifications and DNA methylation in regulating gene expression. For readings on other epigenetic mechanisms please see other reviews (Bannister & Kouzarides, 2011; Cohen et al., 2011; Strahl & Allis, 2000; Sweatt, 2013; Zheng & Hayes, 2003).

Histone modifications.

Eukaryotic DNA is packaged into chromatin that consists of units known as nucleosomes, which is comprised of the DNA double helix wrapping around a protein complex made of eight histone protein subunits. Chromatin can exist in the form of heterochromatin or euchromatin (Tamaru, 2010). In the heterochromatin state, the DNA is tightly packaged which blocks transcriptional machinery and silences gene expression. In contrast, euchromatin is more loosely packaged and permissive of gene transcription.

Histone complexes are protein octamers with 2 copies each of histone H3, H4, H2A, and H2B. Histone variants such as H2A.Z and H3.3 have been associated with replacement of the typical histone proteins during transcription and chromatin structural remodeling (Henikoff et al., 2004). The double-stranded DNA helix is wrapped around the histone octamer ~1.5 times, equivalent to ~147 base pairs, and histone H1 serves as a connector histone between nucleosomes (Thoma et al., 1979). Histones have protruding N- and C-terminal tails in which specific amino acids undergo covalent post-translational modifications, of which the most commonly studied are methylation, acetylation, and phosphorylation (Bode & Dong, 2005; Kouzarides, 2007; Morales et al., 2001).

Histone modifications regulate chromatin structure in various ways, including the recruitment of remodeling enzymes and altering the overall charge of the histone protein complex. Histone phosphorylation state is regulated by kinases and phosphatases that add or remove phosphate groups, respectively, significantly altering the overall charge of the histone which in turn influences chromatin structure. Histone phosphorylation is associated with both transcriptional repression and activation. Histone methylation and acetylation are by far the most studied modifications of these proteins. Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation at lysine (K) residues is generally associated with euchromatin and is permissive of gene expression (Bode & Dong, 2005; Kouzarides, 2007; Morales et al., 2001).

Histone methylation was traditionally thought to be a static process, but it is now widely accepted as being dynamic (Bannister & Kouzarides, 2005; Bannister et al., 2002). Histone methyltransferases (HMTs) regulate the complexity of histone

methylation; lysine (K) residues can be mono-, di-, or tri-methylated and arginine (R) residues can be mono- and di-methylated (either symmetrically or asymmetrically). Methylation is different from acetylation and phosphorylation since it does not change the overall charge of the nucleosome. Unlike acetylation, methylation is associated with both transcriptional activation and repression, which are dependent on various factors including the specific histone subunit, residue, and methylation state. Furthermore, the overall ‘histone code’ or histone ‘crosstalk’ between modifications and DNA methylation makes for an almost infinite level of possible gene expression regulatory processes (Ben-Porath & Cedar, 2001; Kondo, 2009).

DNA methylation.

Most eukaryotic cells contain methylated DNA that is distributed across the genome, referred to as global methylation (Bird, 2002). In mammals, DNA methyltransferases (DNMTs) mostly target a 5' cytosine (C) adjacent to guanine (G) on the same strand (i.e., not complementary C-G base pairing), which is also referred to as a CpG dinucleotide site, although there are exceptions. Regions of DNA with greater than ~55% CpG site content are referred to as CpG islands and tend to appear around promoter regions of genes, with ~40% of genes having CpG islands in their promoter sequences (Miranda & Jones, 2007). It is estimated that the human genome has ~29,000 CpG islands (Bird, 2002). Methylation of CpG sites is associated with gene silencing in cell fate determination, genomic imprinting, and X chromosome inactivation, although many genes with unmethylated CpG sites can also be silenced. DNA methylation is stable and inherited by daughter cells after division even when histone modifications are not, thus maintaining the same gene expression patterns that lead to cell type

determination and perpetuation. DNA methylation represses gene transcription on a multitude of levels: by inhibiting transcription factor access; by attracting methylcytosine-binding proteins (MBDs), one of the most commonly studied being methyl-CpG-binding protein 2 (MeCP2); and by recruiting protein complexes that contain other repressors and chromatin remodelers such as HDACs and HMTs (Jones et al., 1998). DNA methylation may also influence nucleosome occupancy over promoter sites and prevent gene activation (Miranda & Jones, 2007). The notion that DNA methylation patterns in the central nervous system and germ cells are highly dynamic is now widely accepted (Bohacek et al., 2013; Ho & Burggren, 2010; Jiang et al., 2008; Kovalchuk, 2012; Miller & Sweatt, 2007; Roth et al., 2009). Methylation patterns may be “rapid and reversible” by various mechanisms, allowing for previously methylated CpG sites to become de-methylated and vice versa. Several mechanisms of active DNA demethylation have been proposed (Klengel et al., 2014; Ooi & Bestor, 2008). Recently, a large body of evidence has accumulated showing that the epigenetic mechanisms discussed above are involved in drug-induced maladaptive neural plasticity. Below we will briefly summarize the literature concerning psychostimulants and MeCP2.

Addiction and MeCP2

Although drugs of abuse may have different sites of action and affect various neurotransmitter and receptor systems (Taylor et al., 2013), their chronic use leads to specific alterations in gene expression. These changes in gene expression are thought to lead to the changes in behavior, brain structure, and neuronal function that underlie impaired cognition and maladaptive behaviors that are characteristic of addiction (McClung & Nestler, 2008). Rodent and human research has established that multiple

mechanisms of epigenetic regulation are directly affected by drugs of abuse (Biliński et al., 2012; Cadet, 2014). These epigenetic changes may be the primary process by which drugs induce highly stable changes in the expression of genes in multiple brain regions that mediate the transition for casual intermittent drug use to compulsive habitual drug use in addiction. While there are a myriad of known epigenetic changes produced by drugs of abuse, we will primarily focus on MeCP2.

MeCP2 is a methylated DNA binding protein that attracts histone deacetylases (HDACs) and is generally associated with repression of transcription (Jones et al., 1998). It may also act to mediate transcription on a genome wide manner as well (Skene et al., 2010). However, recent studies have shown that MeCP2 can also facilitate transcription in a phosphorylation state-dependent manner (Chahrour et al., 2008; Cohen et al., 2011; Tao et al., 2009). Interestingly, drug exposure mediates levels of MeCP2 in various brain regions and manipulating MeCP2 levels prior to drug exposure can affect the rewarding properties of some psychostimulants (Cassel et al., 2004; Deng et al., 2010; Im et al., 2010). Cocaine self-administration in rats increases MeCP2 mRNA in the anterior cingulate cortex, DS, and NAc (Host et al., 2011). Consistent with this, repeated daily exposure of mice to cocaine injections increased MeCP2 expression in the dentate gyrus, frontal cortex, and DS (Cassel et al., 2006). Im et al. (2010) showed that a lentiviral knockdown of MeCP2 in the DS decreased cocaine intake under extended access conditions. Deng et al. (2010) demonstrated that viral knockdown of MeCP2 in the NAc of mice enhanced amphetamine-induced CPP, whereas overexpression of MeCP2 in the NAc reduced amphetamine CPP. More recently, it has been demonstrated in mice that ethanol exposure modulates MeCP2 expression and inversely that MeCP2 expression is

involved in both ethanol sensitivity and intake behavior (Repunte-Canonigo et al., 2013). Furthermore, ethanol induced changes in MeCP2 expression is a potential molecular mechanism for Fetal Alcohol Spectrum Disorders as continuous ethanol exposure in vitro upregulates MeCP2 expression while ethanol withdrawal down-regulates expression (Liyanage et al., 2015). Hou et al. (2015) conducted a study in which they demonstrated that chronic pain maintains morphine-seeking behavior after morphine withdrawal and these animals had a reduction of MeCP2 at the promoter region of *gria1*, encoding GluA1. Viral overexpression of MeCP2 in the central AMG of these animals eliminated morphine seeking behavior after morphine withdrawal. Additionally, the aforementioned investigations demonstrated that MeCP2 in the NAc is highly related to neuroplasticity as it plays a role in dendritic spine density, GABAergic synapses, and *bdnf* expression regulation (Deng et al., 2010; Im et al., 2010).

Clearly, this collection of studies suggests that MeCP2 plays a role in drug reward and reinforcement. While some studies yielded seemingly conflicting reports, such that virally-mediated reductions in MeCP2 expression in the nucleus accumbens increases the conditioned rewarding effects of amphetamines (Deng et al., 2010), and MeCP2 knockdown in the DS actually reduced cocaine self-administration (Im et al., 2010). This collection of studies suggests that not only is MeCP2 expression regulated by various drugs of abuse, but that MeCP2 expression regulates the sensitivity of an organism, and the reinforcing, and rewarding properties of drugs of abuse. Meaning, that one's individual epigenetic landscape may mediate their physiological and behavioral response to drugs of abuse, therefore altering their predisposition to developing a SUD.

Next, we will briefly review the literature investigating if ELS may be epigenetically enhancing vulnerability toward various psychiatric disorders.

Early Life Stress and Epigenetic Effects

Animal models allow the experimental control and design that are often impractical, impossible, or immoral when using human participants. While peripheral and post-mortem brain samples have been utilized to investigate epigenetic mechanism in humans, animal models allow for the investigation of specific neuronal tissues and circuitries. A recent collection of work, reviewed below, has demonstrated that ELS can in fact modulate multiple brain systems epigenetically. These studies serve as models for the mechanisms by which ELS may be modulating neural systems, such as the reward circuitry, to increase addiction vulnerability. Since much of this literature focuses on the effects of ELS on the hypothalamic-pituitary-axis (HPA) stress response, we will briefly review the neurobiology of this system.

The HPA axis is initiated by either a real or perceived stressful stimulus activating the cells in the paraventricular nucleus (PVN) of the hypothalamus that project to various limbic brain regions. The parvocellular projections of the PVN secrete corticotrophin-releasing factor (CRF, or sometime CRH for corticotrophin-releasing hormone) and arginine vasopressin (AVP) via the median eminence into the anterior pituitary. CRF and AVP in the anterior pituitary stimulate the synthesis and release of adrenocorticotrophic hormone (ACTH) into circulation. ACTH in the blood stream acts on the adrenal cortex to initiate the synthesis and release of glucocorticoids (GC) (primarily cortisol in humans and corticosterone in rodents, collectively referred to as CORT) into circulation. GCs in the systemic circulation then act upon cytosolic and plasma membrane receptors, most

notably glucocorticoid and mineralocorticoid receptors (the latter also known as aldosterone receptors). GCs regulate the termination of the stress response via a negative feedback loop both directly and indirectly in the central nervous system.

Glucocorticoid receptor (*Nr3c1*).

The most extensively studied effect of ELS and epigenetics is of the GC receptor gene (nuclear receptor subfamily 3, group C, member 1, or *Nr3c1*). In the groundbreaking study by Weaver et al., (2004) it was shown that variation in maternal care altered methylation of exon 1₇ of the GC receptor (GR) promoter in hippocampal neurons. More specifically, maternal care was associated with variations in methylation of a specific CpG site in which the immediate early gene nerve-growth factor inducible protein A (NGFI-A) binds DNA as a transcription factor, altering GR expression levels in the hippocampus. A change in GR expression then influences HPA axis function and the negative feedback loop that plays a role in terminating the stress response. This initial research was highly influential, as it demonstrated for the first time a relationship between ELS-altering epigenetic markers regulating protein expression that directly affects neurophysiological systems implicated in psychiatric disorders. Multiple laboratories have continued research investigating the effects of ELS on epigenetic regulation of the *Nr3c1* gene. This avenue of research continues to be promising, although some have failed to replicate Weaver's initial findings (Daniels et al., 2009; Kember et al., 2012). This could be due to different ELS paradigms, or different rodent strains utilized. As others have demonstrated, the effects of ELS on epigenetic regulation of the GR gene is complex and dependent on multiple factors including the genetic background, litter composition, sex, cell type, and brain region of interest (Kember et al.,

2012; Kosten et al., 2013; Kundakovic et al., 2013; Liberman et al., 2012; McGowan et al., 2011). Interestingly, acute and chronic stress in adult rats does not mimic the change in methylation patterns of the *Nr3c1* gene as observed after ELS, demonstrating the importance of the developmental window in this phenomenon (Witzmann et al., 2012).

The similar adult phenotypes observed in rodents and humans with a history of ELS has long suggested that the underlying mechanisms may be conserved across species. Accordingly, McGowan and colleagues were the first to investigate a relationship between ELS and epigenetic regulation of the GR gene in humans, assessing methylation of *Nr3c1* exon 1_F (the human homolog of rat exon 1₇) (McGowan et al., 2009). These investigators found increased DNA methylation at specific CpG sites in exon 1_F of the *Nr3c1* promoter in postmortem hippocampal tissue of suicide victims with a history of ELS compared to controls and suicide victims without a history of abuse. The increased DNA methylation was related to decreased rates of GR expression. Other groups have extended this work by demonstrating a relationship between ELS and methylation status of the *Nr3c1* gene with both increased and decreased methylation states for different GR transcripts (Labonté et al., 2012; Perroud et al., 2011; Steiger et al., 2013; Suderman et al., 2012; Tyrka et al., 2012). The functional impact on the HPA axis and adult behaviors of these epigenetic alterations begs further investigation. While the previous data concerning ELS epigenetically modulating the GR gene is intriguing, McGowan et al. (2011) argued the importance for broadening the investigative scope of this work, as ELS affects numerous systems and behaviors into adulthood. In this latter study, a ~7 million base pair locus around the GR gene in the rat hippocampus was

examined, and findings revealed that maternal care influenced DNA methylation in non-promoter regions in non-random, gene-specific, and bi-directional manners.

Arginine vasopressin.

Arginine vasopressin (AVP) is a hormone involved with HPA regulation, and is also involved in depression (Londen et al., 1997). Murgatroyd et al. (2009) found that mice exposed to ELS had decreased methylation of the *Avp* enhancer and increased AVP expression in the PVN as adults. It is likely that the decrease in methylation in adulthood was due to reduced MeCP2 occupancy at the *Avp* enhancer during ELS. ELS activates HPA circuits which trigger phosphorylation of MeCP2 and its release from methylated DNA. This leads to life-long hypo-methylation and overexpression of AVP in the PVN. This functional consequence of epigenetic regulation was associated with a hyperactive stress response as it was partially reversed by treatment with an AVP V1b receptor antagonist. Additionally, ELS modulates methylation patterns of the *Avp* gene in the hippocampus (Kember et al., 2012) and DNA methylation of other HPA regulatory genes. For example, increased mRNA expression of the pituitary pro-opiomelanocortin (*Pomc*) gene after ELS was associated with a decrease in DNA methylation (Wu et al., 2014). Clearly, the interaction between ELS and epigenetic markers is exceedingly complex, dynamically modulating the HPA axis via multiple avenues in multiple brain regions throughout development.

Brain derived neurotrophic factor.

Brain derived neurotrophic factor (BDNF) is a protein belonging to the neurotrophin family of growth factors, and its gene *bdnf* is another affected by ELS. Roth et al., (2009) used a novel paradigm targeting the effects of abuse during postnatal days 1

– 7. Rat pups were exposed to a stressed female that displayed multiple abuse behaviors including rough physical contact and active avoidance. In adulthood, these rats showed increased DNA methylation of *bdnf* in the PFC and decreased BDNF expression. The DNA methylation status and expression levels of BDNF in the PFC were rescued by chronic intracerebroventricular treatment with a DNMT inhibitor. BDNF has emerged as having somewhat of a ubiquitous role in brain functioning, and is implicated in various diseases including depression, stroke, Alzheimer's, and addictive disorders (Biliński et al., 2012; Nagahara & Tuszynski, 2011). Thus, evidence that ELS can trigger BDNF expression changes in adulthood has immense implications concerning vulnerability towards the development of these disorders.

5-HT transporter (*Slc6a4*).

The serotonin system is highly involved with emotion regulation and various psychiatric illnesses such as major depression, autism, and addictive disorders (Uaswani et al., 2003). Variation in the serotonin transporter (5-HTT) gene (solute carrier family 6, subfamily a, member 4, *Slc6a4*) moderates individual reactions to stress, a potential risk factor for development of psychiatric illness (Caspi et al., 2003; Kendler et al., 2005), and determines clinical responsiveness to certain antidepressants (Kim et al., 2000). ELS influences the development of the serotonergic system, and this regulation may be epigenetically determined (Beach et al., 2010; Vijayendran et al., 2012). In non-human primates, higher levels of methylation of the *Slc6a4* gene has been associated with higher stress reactivity in females exposed to ELS but not controls (Kinnally et al., 2011). Another study conducted in adopted humans demonstrated that DNA methylation levels and the specific 5HTT allele (long or short) interact to influence psychological coping

with loss and trauma (Van IJzendoorn et al., 2010). These data highlight the complex interaction of genetics, epigenetics, and ELS in shaping individual phenotypes.

GABA and glutamate.

ELS also affects the major excitatory and inhibitory neurotransmitter systems, glutamate and GABA, respectively (Bagot et al., 2009; Meaney, 2001) in part via epigenetic mechanisms. Low maternal care was associated with an increase in DNA methylation of the promoter region of the *GAD1* gene in the hippocampus of adult rats (Zhang et al., 2010). The *GAD1* gene codes for glutamic acid decarboxylase (67 kDa isoform), the rate limiting enzyme of GABA synthesis. Methylation status of the *GAD1* promoter in postmortem tissue samples has been associated with schizophrenia (Zhang et al., 2010), implicating a potential mechanism by which ELS may confer vulnerability to this disease. These findings, along with those showing that ELS induced long-term alterations of the GABA_A and central benzodiazepine (CBZ) receptor subunit profiles in the hippocampus (Meaney, 2001), paint an intriguing picture as to the wide scope in which ELS can modify brain systems. Furthermore, ELS epigenetically affects hippocampal long-term-potential and depression, two forms of neural plasticity intimately related to glutamatergic transmission. High maternal care was associated with decreased DNA methylation and changes in histone modifications of the gene coding for the type 1 metabotropic glutamate (mGluR1) receptor (*Grm1*) in the hippocampus of adult offspring (Bagot et al., 2012). Accordingly, these animals displayed an increase in both mRNA and protein levels of the mGluR1 receptor (Bagot et al., 2012). Collectively, these data implicate epigenetic regulation as a mechanism by which ELS can have long lasting effects on excitatory and inhibitory amino acid neurotransmission. Given the

ubiquitous distribution of GABA and glutamate systems across the brain, the ability of ELS to epigenetically modulate their development and function has profound and long reaching implications for resulting adult phenotypes.

Epigenetic machinery.

ELS can alter the expression of epigenetic machinery as well. For example ELS was shown to decrease mRNA levels for multiple HDACs in the cortex (Levine et al., 2012). This decrease in HDAC expression was accompanied by increased acetylation of H4, altered emotional phenotypes, and responsivity to an antidepressant. Other investigators have demonstrated a relationship between ELS and HDAC expression as well (Tesone-Coelho et al., 2013). Additionally, ELS decreased nucleus accumbens DNMT expression rates which were associated with hyper-methylation of neuronal plasticity genes (Anier et al., 2013). It is thus apparent that ELS modulates the epigenome by numerous mechanisms. These alterations can persist into adulthood and regulate gene expression via bi-directional changes in histone acetylation, DNA methylation, and gene expression in both promoter and non-promoter regions (McGowan et al., 2011).

Summary

Thus far, we have reviewed the current literature investigating the long term epigenetically mediated consequences of ELS exposure. Early experience-driven epigenetic markers have the capacity to modulate neuronal gene transcription. Consequent changes in gene expression have been demonstrated to functionally alter multiple neuroendocrine and neurotransmitter systems. In turn, these alterations influence behavior, future responses to various environments, and vulnerability towards multiple psychiatric illnesses, such as addiction. We also discussed the notion that drug induced

epigenetic alterations may be crucial to the process by which casual intermittent drug use transitions to compulsive habitual drug use in addiction (Nielsen et al., 2012; Schmidt et al., 2013; Taylor et al., 2013), while others have shown that the epigenetic landscape prior to drug exposure may play a role in addiction vulnerability (Deng et al., 2010; Im et al., 2010; Tesone-Coelho et al., 2013). There is thus an interesting and circular relationship here, in which the epigenome prior to drug use may predispose the individual to higher addiction vulnerability or resiliency, and the epigenetic response to drugs of abuse further modulates risk and progression towards addiction. Interestingly, despite the established link between ELS and addictive disorders, investigation of ELS induced epigenetic alterations that predispose an individual to a high risk for addiction is still very much in its infancy (Anier et al., 2013; Lewis et al., 2013; Romano-López et al., 2012; Tesone-Coelho et al., 2013). Furthermore, there are contradicting results concerning NAc MeCP2 expression after ELS (Romano-López et al., 2012; Tesone-Coelho et al., 2013). Thus, one possible way by which ELS may be modulating addiction vulnerability is through lasting epigenetic changes in the reward circuitry. Therefore, more research on ELS effects on NAc MeCP2 expression and the role of MeCP2 on the rewarding and reinforcing effects drugs of abuse is warranted.

CHAPTER 2

THE EFFECTS OF MATERNAL SEPARATION ON METHAMPHETAMINE SELF-ADMINISTRATION, EXTINCTION, AND CUE INDUCED REINSTATEMENT

METH is an extremely potent and highly addictive psychostimulant and neurotoxic drug (Xie & Miller, 2009). METH abuse has many detrimental consequences for the individual and for society as a whole. For the individual, chronic abuse has negative neuropsychological and psychiatric effects, and modifies functional and structural reward and learning neurocircuitry in the healthy brain (Taylor et al., 2013). METH abuse has been identified as both a strong risk-factor for violence and high-risk sexual behaviors. In one study of a population between the ages of 18 and 25, 34.9% self-reported violent behavior while under the influence of METH, such as domestic violence, gang-related violence, and random acts of violence (Baskin-Sommers & Sommers, 2006). Individuals on METH often engage in unprotected vaginal and anal sex and also have sex with multiple partners (Springer, et al, 2007). It is apparent that chronic METH use has a multitude of deleterious effects on the users that in turn have a negative outcome on society.

Since METH use has been associated with a variety of detrimental health and social consequences, it is important to identify risk-factors associated with its abuse. Clinical research has shown ELS, particularly childhood abuse and neglect, is a reliable risk factor that influences adult drug abuse (Anda et al., 2006; Messina et al., 2008). Childhood abuse or neglect is highly prevalent with ~1.5 million cases reported in a 2010 survey (Child Maltreatment 2010, US Department of Health and Human Services) and exposure to childhood abuse and household dysfunction has been related to an earlier

onset of METH use in both men and women (Messina et al., 2008). There is substantial evidence that ELS produces long-lasting changes in the brain, including regions that mediate reward-seeking and executive control, which may ultimately predispose the individual to increased propensity toward illicit drug use and addiction (Matthews et al., 2001; Meaney et al., 2002). Stressors during adulthood have also been implicated in affecting drug and alcohol intake behaviors (Piazza et al., 1990; Breese et al., 2011).

The rodent maternal separation model of ELS is a commonly used paradigm to investigate the influences of early life events on addictive behaviors. In this paradigm, rodents undergo daily separation from maternal care during critical postnatal development and are assessed for propensity towards addiction-like behaviors in adulthood. For example, pups undergoing maternal separation for several hours exhibit depression-like symptoms, high anxiety-like behavior, exaggerated neuroendocrinological responses to stress, and have a high preference for ethanol (Huot et al., 2001). MS has been reported to also alter the reinforcing effects of cocaine, amphetamine, and morphine (Der-Avakian & Markou, 2010; Moffett et al., 2006; Vazquez et al., 2005). However, only a few reports have been published on the effects of maternal separation on adult METH vulnerability. In one study, maternal separation failed to produce a significant increase in adolescent METH CPP (Faure et al., 2009), while another study demonstrated that maternal separation attenuated METH CPP in adolescents (Dimatelis et al., 2012b). Maternal separation has also been shown to produce a sex- and dose-dependent increase in locomotor and stereotypy responses to METH in adolescent rats (Pritchard et al., 2012). To our knowledge, however, there are no reports to date on the effect of maternal separation on adult intravenous METH SA,

extinction, and reinstatement. Given the negative impact of METH abuse and the relationship observed between maternal separation and other drugs of abuse, more research in this area is warranted.

Emerging evidence suggests a strong role of epigenetics in regulating gene transcription based on early experiences that in turn modulate brain systems and behavior into adulthood. Many neural systems implicated in drug addiction are influenced by MS, such as the HPA axis (Plotsky and Meaney, 1993), endocannabinoid system (Romano-Lopez et al., 2012), monoaminergic systems (Matthews et al., 2001, Ploj et al., 2003; Dimatelis et al., 2012), and growth factors such as BDNF (Bolaños & Nestler, 2004, Lippmann et al., 2007). Recent studies implicate epigenetic modifications as a mechanism behind these changes in rodents, non-human primates, and humans (McGowan et al., 2009; Murgatroyd et al., 2009; Kinnally et al., 2011). For example, Murgatroyd (2009) showed that maternal separation induced hypomethylation of the AVP enhancer, subsequently causing upregulation of Avp expression and a hyper-responsive HPA axis. Additionally, maternal care has been implicated in DNA methylation and corresponding changes in glucocorticoid receptor (GR) expression levels in the hippocampus (Weaver et al., 2004). Furthermore, adult rats exposed to ELS have demonstrated reduced BDNF in the PFC correlated with hypermethylation of the BDNF IV promoter region (Roth et al., 2009). Indeed, these studies suggest that early life experiences are influencing epigenetic markers that modulate multiple brain systems implicated in drug vulnerability.

Interestingly, epigenetic factors are also altered by drug exposure and can influence drug intake, behavioral, and neural responses (Lewis & Olive, 2013; Biliński et

al., 2012; Cadet, 2014; Renthal & Nestler, 2009). For example, trimethylation of histone H3 lysine 4 (H3K4) at the promoter region of a chemokine receptor type 2 (CCR2), a gene implicated in locomotor sensitization, has been associated with METH-induced hyperlocomotion in mice (Ikegami et al., 2010). Additionally, cocaine increases MeCP2 expression in multiple brain regions of the rat (Cassel et al., 2006) and MeCP2 has been implicated in cocaine and amphetamine reward and reinforcement (Im et al., 2010; Deng et al., 2010). Specifically, Deng et al (2010) found that virally-mediated ablation of MeCP2 expression in the NAc increased the conditioned rewarding effects of amphetamines, whereas overexpression of MeCP2 in the NAc decreased amphetamine reward. Furthermore, Im et al (2010) showed that cocaine intake was reduced after knockdown of MeCP2 expression in the DS. Hence, recent studies suggest that the predisposition of one's epigenetic phenotype may influence their behavioral response to drugs of abuse while exposure to drugs of abuse also modulates their epigenetic phenotype.

Although maternal separation and psychostimulants have been shown to individually affect epigenetic factors such as MeCP2, and MeCP2 has been implicated in drug seeking behavior, it is yet to be determined if maternal separation and, specifically, METH also interact to affect epigenetic factors. Therefore, the goal of the present study was to investigate the relationship between ELS, METH SA, extinction and reinstatement, and MeCP2 expression in the NAc. We hypothesized that MS would increase susceptibility to the acquisition of METH SA, impair extinction learning, and increase cue-induced reinstatement. We also predicted that MeCP2 expression in the NAc would be negatively correlated with levels of METH SA.

Abbreviated Methods

Animals

Pregnant dams were purchased from Charles River Laboratories and arrived on gestational day 12. Litters were randomly assigned to 1 of 2 conditions: maternal separation for 180 min per day (MS180) or the handled control group, 15 min per day (MS15). Separation procedures were carried out from PND2-14. During PND15-20 litters were left undisturbed, weaned on PND21 into same sex group housing, and pair housed with a sibling on PND45. Females were not used for the remainder of the study. Male rats were implanted with intravenous catheters into the jugular vein on PND60 (± 1).

Behavioral Testing

Beginning on PND67 male rats underwent 2 hr daily SA sessions whereby presses on one of the levers (designated the active lever) resulted in delivery of METH (0.05 mg/kg per infusion, delivered in a volume of 0.06 ml over a 2 sec period) on an fixed ratio 1 (FR1) schedule of reinforcement. Each METH infusion delivery was followed by a 20-sec timeout period, during which additional active lever presses were recorded but produced no drug infusions. Each infusion was accompanied by concurrent illumination of a stimulus light located directly above the active lever, and presentation of an auditory stimulus for 2 sec. SA sessions were conducted 7 days per week for 15 consecutive days. Next, all animals were subjected to 2 hr extinction training sessions for 15 consecutive days, whereby presses on the active lever no longer produced any programmed consequences. On the day immediately following the last extinction session, all rats underwent cue-induced reinstatement, whereby presses on the active lever produced the tone and light cue previously presented during METH infusion, but did not deliver any

drug solution. Presses on the inactive lever did not produce any programmed consequences throughout the experiment.

Tissue Preparation, Immunohistochemistry, and Analysis

Fluorescent immunochemistry procedures for labeling MeCP2 were carried out according to standard procedures. A total of three bilateral slices of the NAc core were counted for each subject (i.e., 1 sample area/2 hemispheres/3 sections). The counts from all six sample areas from the NAc core were averaged to provide a mean number of immunoreactive cells per animal to be used as an $n = 1$ for statistical analysis.

Results

Litter Effects

A repeated-measures ANCOVA conducted by litters, controlling for rearing condition, revealed no significant pre-existing differences between litters on the number of METH infusions per session over 15 days ($p = 0.23$).

METH Self-Administration

Repeated-measures ANOVA revealed a significant main effect of rearing condition on the number of METH infusions per session, $F(1, 24) = 9.83, p = 0.004$ (Figures 1 and 2), as well as the number of total active lever presses per session, $F(1, 24) = 13.79, p = 0.001$ (data not shown), with MS180 having more active lever presses and receiving more infusions than MS15. No group differences in the total number of inactive lever presses were observed, $F(1, 17) = 38.76, p = 0.425$. However, in both rearing conditions we noted a time-dependent increase in inactive lever pressing across self-administration sessions (see Table 1), and we attribute this to be a result of non-specific motor activity that resulted from increasing level of METH SA.

Extinction

For both groups, extinction training produced a significant reduction in the number of active lever presses when comparing the average of the final 2 days of extinction to the average of the final 2 days of SA ($n = 51$), $t(50) = 5.10$, $p < 0.0001$. Repeated measures ANOVA revealed no significant group differences (MS15: $n = 9$, MS180: $n = 17$) in rate of extinction of active lever pressing, $F(1, 20) = 0.94$, $p = 0.34$ (Figure 3). However, a significant group difference in the number of inactive lever presses during extinction training $F(1, 24) = 5.47$, $p = 0.028$, was observed, with rats in the MS15 group emitting more inactive lever presses over the 15 day extinction period compared to the MS180 group (see Table 1).

Cue-Induced Reinstatement

Cue induced reinstatement was observed in all animals as assessed by the number of active lever presses (averaged across the final 2 days of Ext) compared to active lever presses during the reinstatement session, $t(50) = -4.46$, $p < 0.0001$. However, there was no significant difference between the groups for the number of active lever presses during reinstatement testing, $F(1,24) = 1.134$, $p = 0.298$ (Figure 4).

MeCP2 Immunoreactivity

A total of 6 pups from 5 different litters (3 per rearing condition) were used in the analysis of the MeCP2 data. We found a significant difference in MeCP2 immunoreactivity between MS15 rats and MS180 rats in the NAc core, $p < 0.001$, with MS15 expressing more labeled profiles than did MS180 (Figure 5). There was also a negative correlation between MeCP2 immunoreactivity and number of total active lever presses during 15 days of SA, $r = -0.839$, $p = .003$ ($n = 5$ per rearing condition), (Figure

6). Rats emitting fewer lever presses expressed higher numbers of labeled profiles in the NAc core (Figure 7).

Discussion

Early life maternal care is known to influence a multitude of neurological, endocrine, epigenetic, and behavioral outcomes in adulthood (Roth, 2012; Francis et al., 1999). Our findings contribute to the literature by suggesting that MS causes alterations that influence vulnerability to drug abuse (Moffett et al., 2007), in this case METH SA. For the first time, our study suggests that either repeated and prolonged maternal separation leads to increased vulnerability to METH intake or that minimal maternal separation protects against adult METH SA vulnerability. This is evidenced by our findings that MS180 rats showed higher levels of METH SA over 15 daily sessions compared to MS15. These findings are in agreement with previous studies examining effects of maternal separation on intake of cocaine, morphine, amphetamine, and ethanol (Moffett et al., 2006; Der-Avakian et al., 2010; Vazquez et al., 2006; Huot et al, 2000). Additionally, we noted that MS15 rats demonstrated a preference for the inactive lever over the active lever during SA. While the reason for this is currently unknown, a possible explanation for this counterintuitive observation is different non-specific behavioral response to METH SA or enhanced operant sensation seeking in the MS15 group.

Since our data set does not include a non-handled control it is difficult to distinguish whether the SA behavior is reflective of increased vulnerability in the MS180, protective effects in MS15, or both. We chose MS15 as the ideal control group since it controls for handling effects, while other commonly used control groups do not.

Furthermore, the use of control groups (including MS0, non-handled, and Animal Facility Reared) is highly variable. The issues concerning different control groups and variations in procedures have previously been discussed by others (Matthews et al., 2001; Matthews et al., 1999). Jaworski et al. (2005) provides a well laid out table comparing different experimental and control groups commonly used. Recently, a trend towards comparing only two groups has emerged. For example, Matthews (2001) used a MS2 and MS360, Ploj (2003) only used MS15 and MS360, and Murgatroyd (2009) used non-disturbed and MS180 with mice. Our current paradigm met the goal of optimizing the differences between conditions and is consistent with the type of two group design that is currently gaining momentum in this field. The current literature on maternal separation and drug reward, reinforcement, and SA demonstrates that MS180 and MS15 tend to be the most divergent groups when compared to the various controls. For this reason, in the present study, we did not include a non-separated control group in order to increase validity and reliability in our data and improve interpretation in comparison with other studies. Additionally, there are large inconsistencies across laboratories with regards to procedures for breeding, culling, fostering, litter sex ratios, separation duration and days, the order in which dams and pups are returned to the home cage, controlled temperature settings outside of the home-cage, PND of weaning, and post-weaning housing conditions prior to and during manipulations.

The effects of brief and prolonged maternal separation we found on METH SA fits the inverted U-shape typically found in drug abuse-related behaviors after maternal separation (Neisewander et al., 2012). It has been postulated that the protective effects seen in the MS15 group may be due to the increased maternal care post separation

(Francis & Kuhar, 2008; Marmendal et al., 2004). Many have argued that the MS15 rearing condition is more ethologically relevant than the standard non-separated controls since food foraging and other activities would necessitate the dam to leave the litter for brief amounts of time. The possible protective or resilient effect in the MS15 group provides an interesting comparison. MS15 adults have shown reduced responding for cocaine when compared to non-separated controls (Flagel et al., 2003; Moffett et al., 2006).

For almost a decade, it has been known that maternal care during early neurological development influences DNA methylation that is directly responsible for HPA reactivity to stress throughout the lifespan. Weaver et al. (2004), showed that offspring of low licking/grooming and arch-back nursing (LG-ABN) mothers had higher levels of GR DNA methylation, decreased expression of the GR gene, a heightened HPA stress response, and displayed more fear-like behavior. Since this pioneering study, many laboratories have demonstrated various alterations in DNA methylation in adulthood following ELS. For example, ELS has been associated with increased global methylation, as well as increased methylation at the regulatory region of serotonin transporter (5-HTT), and higher behavioral stress responses in female macaques (Kinnally et al., 2011). ELS has also been found to induce hypomethylation of the AVP enhancer in male mice with a subsequent increased HPA reactivity (Murgatroyd et al., 2009). Although the brain region, gene, and direction in which DNA methylation is altered by ELS is diverse, the outcome tends to remain constant, with a hyperactive HPA stress response and/or increased behavioral stress reactivity in adulthood. Since an overactive HPA axis and ELS are strongly associated with a higher risk for drug addiction, additional research is

needed to investigate if ELS mediates epigenetic factors influencing the reward network that may predispose the animal to a higher propensity towards drug intake.

MeCP2 is a methylated DNA binding protein that attracts HDACs and is commonly associated with specific gene silencing and repression of transcription (Jones et al., 1998), although it may also act to mediate transcription on a genome wide manner as well (Skene et al., 2010). Interestingly, drug exposure mediates levels of MeCP2 in various brain regions and manipulating MeCP2 levels prior to drug exposure can affect the drugs rewarding properties (Cassel et al., 2004; Im et al., 2010; Deng et al., 2010). Therefore we investigated the MeCP2 levels in the NAc core, a brain region associated with the initial rewarding effects of drugs of abuse (Taylor et al., 2013). We observed group differences in MeCP2 immunoreactivity in the NAc core, such that MS15 rats expressed significantly higher levels of MeCP2 compared to MS180 rats.

Our results suggest a difference in DNA methylation in the NAc; however, the precise genes where methylation has occurred and that is bound by MeCP2 was not determined. Previous studies have suggested that MS rats may have altered DA, NE, and 5-HT function and GABA and glutamate levels in the NAc (Matthews et al., 2001; Romaro-Lopez et al., 2012). It has also been demonstrated that nucleus accumbens protein expression is extensively changed after both MS and METH exposure (Dimatellis et al., 2012). Therefore, the difference in methylated DNA may be associated with any number of genes involved in these systems in the NAc, and identification of methylated genes is worthy of further investigation. It is important to note that Romaro-Lopez (2012) did not find a difference in MeCP2 levels in the NAc between their separated and non-separated pups using immunoblotting techniques. Thus, quantification by

immunohistochemistry may not reveal the same results as by immunoblotting.

Additionally, the differences in separation procedures and drug exposure potentially played a role in these contrary results.

The negative correlation between active lever presses and MeCP2 immunoreactivity in the NAc core aligns well with Deng et al. (2010) in which MeCP2 in the NAc had an inverse relationship with amphetamine CPP. This data warrants future investigations in order to explicate this relationship. For example, additional studies are needed to determine the influence of rearing condition on MeCP2 levels in the NAc in drug-naïve animals as well as the influence of varying levels of METH exposure. Also worthy of future studies is the possibility that an enriched environment during adolescence could reverse the detrimental effects of maternal separation on METH SA in adulthood and also mediate MeCP2 expression in the NAc. Enriched environment during an abstinence phase of cocaine showed protective effects to cue-induced reinstatement (Thiel et al., 2009) and reduced CPP to cocaine (Solinas et al., 2008). More recently, it was demonstrated that an enriched environment during different developmental time points can protect against METH SA acquisition and cue-induced reinstatement (Lü et al., 2012).

Few studies have investigated the effect of maternal separation on drug relapse paradigms yet, there some data that suggests ELS may increase relapse vulnerability (Neisewander et al., 2012). Contrary to existing literature and our predictions that maternal separation would influence extinction rates and cue-induced reinstatement, we failed to detect an effect. It is possible that we may have detected an extinction or reinstatement effect if the rats were trained on a progressive ratio or a higher FR of

reinforcement since these schedules produce higher response rates. On the other hand, failing to find an effect may be indicative that rearing condition only influenced the initial rewarding or reinforcing effects of METH as opposed to the subsequent course of addiction, abstinence, and relapse. Also, we only tested for cue-induced reinstatement, future research is necessary to determine group differences in stress and drug induced reinstatement.

In summary, we observed that ELS in the form of extended maternal separation produced an increased vulnerability to adult METH SA in adult male rats. Increases in METH intake were paralleled by decreased MeCP2 immunoreactivity in the NAc core. Surprisingly, extinction and cue-induced reinstatement were unaffected by maternal separation. These results suggest the possibility that ELS may contribute to vulnerability towards METH intake. Further studies are needed to establish a contributory role for changes in MeCP2 levels in the NAc core or other brain regions in these behavioral effects.

CHAPTER 3

EARLY LIFE STRESS INFLUENCES METHAMPHETAMINE INTAKE BEHAVIOR THROUGH MODIFICATION OF MECP2 EXPRESSION IN THE NUCLEUS ACCUMBENS CORE

MeCP2 is a multifunctional epigenetic protein well known for binding to methylated CpG islands. DNA bound MeCP2 recruits transcriptional repressor complexes such as Sin3a, HDAC1 and 2 (Jones et al., 1998), creating a barrier to transcriptional machinery and silencing downstream genes. Although, MeCP2 interacts with DNA on multiple complex levels. For instance, MeCP2 also has binding sites for both un-methylated DNA and chromatin. Interactions at these sites can lead to DNA bridging and looping, chromatin condensation, and nucleosomal clustering (Hansen et al., 2010; Nikitina et al., 2007), all which regulate transcription. Additionally, MeCP2 can bind to multiple cytosine variants, such as a methyl group attached to the carbon 5 position of cytosine to produce 5-methylcytosine (5-mC) and to the oxidized 5-methylcytosine into 5-hydroxymethylcytosine (5-hmC) with similar high affinities in the brain (Mellén et al., 2012). While bound to 5hmC, MeCP2 may act as a transcription facilitator. The interactions between MeCP2 and DNA are diverse, leading to various functional outcomes, making it a viable protein to study for various disease states.

The role dynamic epigenetic architecture plays in vulnerability and development of psychiatric disease has received an increasing amount of attention over the last couple decades. In 1999, a report pinpointed 6 mutations in the gene coding for MeCP2 as the cause for Rett Syndrome, an X-linked neurological disorder causing intellectual disability. Mutations in the gene coding for MeCP2 have also been implicated in other

disorders such as MeCP2 Duplication Disorder, Angelman Syndrome, X-linked Intellectual Disability, and Severe Neonatal Encephalopathy (see Ezeonwuka & Rastegar, 2014 for review). MeCP2 plays a role in the neurodegenerative disorder Huntington's disease through interaction with huntingtin protein to regulate transcription of BDNF (McFarland et al., 2014). Animal models have also demonstrated that MeCP2 is involved in the molecular, cellular, and behavioral responses to drugs of abuse.

Drug addiction, or SUD, is a product of chronic exposure to drugs of abuse that modulate gene expression, ultimately altering brain reward circuitry. Therefore, drug induced changes in the epigenome, such as MeCP2 expression, of neuron populations in brain reward regions has been postulated as a mechanism in the development of these disorders. 5-HT modulating drugs such as fluoxetine and cocaine were shown to increase MeCP2 expression in multiple brain regions of the rat after 10 days of investigator-delivery (Cassel et al., 2006) and after 4 days of intravenous cocaine SA (Host et al., 2011). In 2010, two seminal papers implicated striatal MeCP2 in the reinforcing and rewarding effects of amphetamine and cocaine through CPP and SA paradigms (Deng et al., 2010; Im et al., 2010). Additionally, MeCP2 regulated dendritic spine density, the number of GABAergic synapses, and the expression of *bdnf* via homeostatic interactions with microRNA-212 (Deng et al., 2010; Im et al., 2010), suggesting that MeCP2 may be intimately involved in the maladaptive plasticity implicated in the transition from casual to compulsive drug use. More recently, it has been demonstrated in mice that ethanol exposure modulates MeCP2 expression and inversely that MeCP2 expression is involved in both ethanol sensitivity and intake behavior (Repunte-Canonigo et al., 2013). Furthermore, ethanol induced changes in MeCP2 expression is a potential molecular

mechanism for Fetal Alcohol Spectrum Disorders as continuous ethanol exposure *in vitro* upregulates MeCP2 expression while ethanol withdrawal down-regulates expression (Liyanage et al., 2015). Hou et al. (2015) recently conducted a study in which they demonstrated that chronic pain maintains morphine-seeking behavior after morphine withdrawal and these animals had a reduction of MeCP2 at the promoter region of *grial*, encoding the GluA1 receptor subunit. Viral overexpression of MeCP2 in the central AMG of these animals eliminated morphine seeking behavior after morphine withdrawal. This collection of studies suggests that not only is MeCP2 expression regulated by various drugs of abuse, but that MeCP2 expression regulates the sensitivity of an organism, and the reinforcing and rewarding properties of drugs of abuse. Meaning, that one's individual epigenetic landscape may mediate their physiological and behavioral response to drugs of abuse, therefore altering their predisposition to developing a SUD.

The link between ELS and psychopathology, including SUDs, is well documented. Exposure to ELS alters neurobiological development by programming various systems such as the neuroendocrine stress response, the sympathetic nervous system, monoamines, oxytocin, and the immune system to name a few (De Bellis & Zisk, 2014). While the last century has produced this impressive body of knowledge, the mechanism by which experiences early in life modulate complex neurobiological systems and behavior throughout adulthood remained elusive until the last decade. Weaver et al. (2004) demonstrated that maternal care during early life in the rat led to persistent DNA methylation changes along with altered behavioral and neuroendocrine phenotypes in adulthood. Specifically, they showed that high maternal care, defined as licking/grooming and arch-back nursing (LG-ABN), led to increased GR expression in

the hippocampus when compared with pups reared by low LG-ABN dams. The higher GR mRNA expression was associated with lower DNA methylation in the promoter region of the GR gene, thereby facilitating the binding of the transcription factor nerve growth factor-induced protein A (NGFI-A). Interestingly, since this seminal paper, MeCP2 has been shown to contribute to ELS-dependent epigenetic programming of HPA regulatory genes (CRH, AVP, and Pomc) in stress circuitry brain regions (Murgratroyd et al., 2009; Wang et al., 2014; Wu et al., 2014). Clearly, ELS is epigenetically designing adult stress circuitry resulting in altered physiological and behavioral phenotypes.

While this literature demonstrates that ELS is epigenetically programming stress circuits, few studies have investigated if ELS is epigenetically programming reward circuitry implicated in the development of SUDs. Recent studies found contradicting results concerning NAc MeCP2 expression after ELS (Romano-Lopez et al., 2012; Tesone-Coelho et al., 2013). In our previous study (Lewis et al., 2013), we found that ELS increased METH intake behavior compared to handled control animals. A history of ELS and high METH intake was related to less NAc core MeCP2 expression in these animals. From this study, we were unable to determine what was modulate striatal MeCP2 expression in these animals. Therefore, we set out to determine if ELS and METH SA, independently, and/or interact to modulate MeCP2 expression in various brain reward regions. It has previously been shown that virally mediated knockdown of DS MeCP2 decreases cocaine intake behavior (Im et al., 2010) and knockdown of NAc MeCP2 increases amphetamine CPP (Deng et al., 2010). However, since the progression of initial drug use to compulsive drug abuse entails a transition from the NAc to the DS, we investigated if virally mediated knockdown of MeCP2 in the NAc core influenced

METH intake behavior. We also investigated if this manipulation would affect intake behavior for a non-drug reinforcer, saccharin (sacc). Specifically, we predicted that both ELS, in the form of prolonged maternal separation, and METH SA would increase MeCP2 in brain reward regions. Furthermore, we predicted that exogenously reducing MeCP2 expression in the NAc core would decrease METH intake, motivation, and seeking behavior.

Abbreviated Methods

Study 1

This study was designed to investigate the influence of ELS, in the form of prolonged maternal separation, and METH SA on MeCP2 in various brain regions. One to two pups per litter were used for statistical analysis.

Animals

Pregnant dams were purchased from Charles River Laboratories and arrived on gestational day 12. Litters were randomly assigned to one of two conditions: maternal separation for 180 min per day (MS180) or the handled control group, 15 min per day (MS15). Separation procedures were carried out from PND2-14. During PND15-20 litters were left undisturbed, weaned on PND21 into same sex group housing, and pair housed with a sibling on PND45. Females were not used for the remainder of the study. Male rats were implanted with intravenous catheters into the jugular vein on PND60 (± 1).

Behavioral Testing

Beginning on PND67 male rats underwent 2 hr daily SA sessions whereby an active nose poke operant response resulted in delivery of METH (0.05 mg/kg per infusion, delivered in a volume of 0.06 ml over a 2 sec period) on a FR1 schedule of

reinforcement. Each METH infusion delivery was followed by a 20-sec timeout period, during which additional active responses were recorded but produced no drug infusions. Each infusion was accompanied by concurrent illumination of a stimulus light located directly above the active nose poke, and presentation of an auditory stimulus for 2-sec. See Appendix General Methods for methods concerning transcardial perfusion and immunohistochemistry.

Study 2

This study was designed investigate the influence of NAc core MeCP2 expression on METH and sacc intake, motivation, and reinstatement.

Animals

Male Long Evans rats were purchased from Charles River Laboratories and arrived on PND45. Rats were allowed to acclimate to the new environment for 5-7 days before viral infusion surgeries. An adeno-associated virus (AAV) expressing a short hairpin loop RNA (shRNA) against rat MeCP2 mRNA (mecp2-sh) or an empty control virus (mecp2-ctrl) were synthesized by Virovek Inc (Hayward, CA) was infused in a volume of 2 μ l per side (2.25×10^{13} particles/ml) (See Appendix). Animals were left undisturbed for 8 days before receiving jugular vein catheter implantations (See Appendix). Animals were allowed 5 days of recovery before beginning SA procedures at approximately PND65.

Behavioral Testing

Male rats underwent 6 hr daily SA sessions whereby an active nosepoke operant response resulted in delivery of METH (0.05 mg/kg per infusion, delivered in a volume of 0.06 ml over a 2 sec period) on a FR1 schedule of reinforcement. After earning 10 or

more infusions in two consecutive days, rats progressed to FR3 and then FR5 schedules of reinforcement. Each METH infusion delivery was followed by a 20-sec timeout period, during which additional active responses were recorded but produced no drug infusions. Each infusion was accompanied by concurrent illumination of a stimulus light located directly above the active nose poke, and presentation of an auditory stimulus for 2 sec. After self-administration, animals underwent 2 days of progressive ratio testing, in which the number of lever presses required to obtain an infusion of METH increased exponentially (Richardson and Roberts, 1996). Next, lever responding was re-established by two days METH SA sessions on an FR1 schedule. Following reestablishment, rats underwent extinction training for 8 days, where all operant responses produced no programmed consequences. Following extinction, METH rats were administered a 1 mg/kg intraperitoneal (i.p.) priming injection of METH 30 min prior to placement into the operant chamber. Sacc trained rats were placed into the operant chamber and underwent cue-induced reinstatement.

See Appendix B, I, and L for methods on Rotarot testing, virus design, bilateral virus injection surgeries (Figure 8), and virus validation via immunoblot analysis.

Results

Study 1

Self-administration.

Repeated-measures ANOVA revealed a significant main effect of rearing condition on the number of METH infusions per session, $F(1, 16) = 10.88$, $p = .005$ (Figure 9), but no within-effect of days, $F(1, 16) = .17$, $p = .69$. Repeated-measures ANOVA revealed a significant main effect of rearing condition on the active responses

per session, $F(1, 16) = 9.59$, $p = .007$ (see Figure 10), but no within-effect of days, $F(1, 16) = .93$, $p = .35$. The Early Life Stress (ELS) group exhibited more active responses and received more infusions than the Handled Control (HC) group. Repeated-measures ANOVA revealed no significant difference in inactive responses between groups, $F(1, 16) = .43$, $p = .52$.

Immuno-labeling MeCP2 analysis.

A 2x2 multi-analysis of variance (MANOVA) was used to investigate the effects of ELS (HC Saline vs ELS Saline) and METH SA (HC METH vs HC Saline) on MeCP2 immunoreactive labeling in the: nucleus accumbens core, NAc core $F(3, 23) = 4.89$, $p = 0.01$, LSD post hoc analysis HC METH vs HC Saline, $p = 0.001$, ELS METH vs ELS Saline $p = .8$, HC Saline vs ELS Saline, $p = 0.01$ (Figure 11 and Figure 12); NAc shell, NAc shell $F(3, 23) = 3.98$, $p = 0.02$, LSD post hoc analysis HC METH vs HC Saline, $p = 0.01$, ELS METH vs ELS Saline $p = 0.9$, HC Saline vs ELS Saline, $p = 0.15$ (Figure 13); the medial dorsal striatum, $F(3, 23) = 5.86$, $p = 0.005$, LSD post hoc analysis HC METH vs HC Saline, $p = 0.005$, ELS METH vs ELS Saline $p = 0.01$, HC Saline vs ELS Saline, $p = 0.56$ (Figure 14); and the lateral dorsal striatum, $F(3, 23) = 3.29$, $p = 0.04$, LSD post hoc analysis HC METH vs HC Saline, $p = 0.02$, ELS METH vs ELS Saline $p = 0.11$, HC Saline vs ELS Saline, $p = 0.11$ (Figure 15). The n's per group were as follows, HC Saline: $n = 5$, HC METH: $n = 5$, ELS Saline: $n = 6$, and ELS METH: $n = 7$.

Study 2

METH.

Self-administration.

Repeated-measures ANOVA revealed a significant main effect of sh-MeCP2 on the number of METH infusions per session, $F(1, 15) = 16.77$, $p = 0.001$ (Figure 16), and a significant within-effect of days, $F(1, 15) = 6.32$, $p = 0.02$. The sh-Control group received more infusions than the MeCP2 KD group. Repeated-measures ANOVA revealed no significant difference in inactive responses between groups, $F(1, 12) = 1.57$, $p = 0.23$.

Progressive ratio.

The sh-MeCP2 group demonstrated reduced motivation for METH in the progressive ratio paradigm for active lever presses compared to sh-Control, $t(13) = 3.56$, $p = 0.003$ and for reinforcements $t(13) = 4.49$, $p = 0.001$ (Figures 17 and 18).

Extinction and drug-primed reinstatement.

For both sh-Control and sh-MeCP2 groups, extinction training produced a significant reduction in the number of active responses as assessed on the final two days of extinction as compared to the final two days of SA (p 's < 0.05) (Figure 19). For both groups, METH-priming produced reinstatement, as evidenced by a significant increase in the number of active responses following METH administration versus the average of the last 2 days of Ext (p 's < 0.05) (Figure 19). The groups did not differ in their reinstatement magnitude (calculated by a ratio of reinstatement to SA active responses), $t(13) = 1.12$, $p = .14$.

Saccharin

Self-administration.

Repeated-measures ANOVA revealed a significant main effect of sh-MeCP2 on the number of Sacc reinforcers per session, $F(1, 8) = 6.52$, $p = 0.034$ (Figure 20), and a significant within-subjects effect of days, $F(1, 8) = 22.49$, $p = 0.001$. The Control group received more Sacc reinforcers than the MeCP2 KD group. Repeated-measures ANOVA revealed no significant difference in inactive responses between groups, $F(1, 8) = 1.61$, $p = 0.24$, nor over time, $F(1, 8) = 0.18$, $p = 0.68$.

Progressive ratio.

The sh-Control and sh-MeCP2 groups did not differ in motivation for sacc in the progressive ratio paradigm for active lever presses ($p = 0.9$) or reinforcements ($p = 0.7$) (Figures 21 and 22).

Extinction and cue-primed reinstatement.

For both sh-Control and sh-MeCP2 groups, extinction training produced a significant reduction in the number of active responses as assessed on the final two days of extinction as compared to the final two days of SA ($p = .01$ and $p = .023$ respectively) (Figure 23). For both groups, cue-priming produced reinstatement, as evidenced by a significant increase in the number of active responses following cue priming versus the average of the last two days of extinction ($p = .031$ and $p = .002$ respectively) (Figure 23). The groups did not differ in their reinstatement magnitude (calculated by reinstatement/SA active responses), $t(8) = 0.29$, $p = .35$.

Rotational motor activity.

We failed to find a difference between sh-Control and sh-MeCP2 (n = 12, 6/group) on rotational motor activity measured in 360°, 90°, and 2° turns (all p 's > .5) (Figure 24, A). Additionally, we failed to find a difference between sh-Control and sh-MeCP2 (n = 15, sh-Control: n = 6, sh-MeCP2: n = 9) on METH primed rotational motor activity measured in 360°, 90°, and 2° turns (all p 's > .5) (Figure 24, B).

Immunoblot analysis.

Differences in MeCP2 levels across groups were present in the NAc core. Animals who received bi-lateral injection of sh-MeCP2 had significantly decreased MeCP2 levels compared to animals who received bi-lateral injection of sh-Control (ns = 4/group), $p = .001$. There was no group differences in levels of the loading control, GAPDH (Figure 25).

Discussion

In this study, we show molecular and behavioral evidence that ELS increases METH intake behavior in male rats through modulation of NAc core MeCP2 expression. Our results suggest that the experience of ELS has long-lasting effects on MeCP2 expression in NAc core and expression in this region plays a role in METH intake behavior. We show METH self-administration increases MeCP2 expression throughout the striatum in an ELS dependent manner. Furthermore, we demonstrate that virally mediated knockdown of NAc core MeCP2 expression drastically decreases METH intake behavior, suggesting that NAc core MeCP2 expression may be necessary for escalating METH intake. This highlights a possible epigenetic mechanism by which ELS influences vulnerability towards increasing METH intake. During the past decade, a large body of

evidence demonstrating that ELS epigenetically alters the HPA axis has emerged; here we present evidence of ELS affecting brain reward circuitry via altered NAc core MeCP2 expression.

MeCP2 is an epigenetic transcriptional regulator and multifunctional protein that can activate or repress transcription of downstream genes (Chahrour et al., 2008). Interestingly, genes regulated by MeCP2 are involved in drug reward and reinforcement (Deng et al., 2010; Deng et al., 2014; Hou et al., 2015; Im et al., 2010; Jayanthi et al., 2014; Zhang et al., 2014;). For example, MeCP2 is implicated in opioid seeking behavior in rats after persistent pain through regulating GluA1 proteins in the central amygdala. Additionally, MeCP2 represses the histone dimethyltransferase G9a in the central amygdala, leading to an increase in BDNF expression that increases morphine conditioned reward (Zhang et al., 2014). These same investigators found that knock down of MeCP2 in the DS reduced cocaine intake and identified MeCP2 as an indirect regulator of BDNF through interactions with microRNA-212 (Im et al, 2010). Chronic METH exposure induced a reduction of AMPA receptors in striatal neurons is in part mediated by MeCP2 recruitment (Jayanthi et al., 2014). Furthermore, psychostimulant induced phosphorylation of MeCP2 regulates behavioral responses to both amphetamine and cocaine (Deng et al., 2010; Deng et al., 2014). In line with these studies, the present study demonstrates that ELS increases MeCP2 expression in the NAc core, a brain region implicated in drug reward and reinforcement. Importantly, we also show that viral knockdown of MeCP2 in this region decreases METH intake and motivation behavior in male rats.

In this study, we replicated our previous findings (Lewis et al., 2013; Chapter 2) that ELS, in the form of prolonged maternal separation, increases METH intake behavior compared to handled controls in male Long-Evans rats. Here, we also demonstrate that ELS increases MeCP2 expression in the NAc core but not the NAc shell or the DS. Additionally, we show that METH SA increases MeCP2 expression throughout the ventral and DS in an ELS experience-dependent fashion. Such that METH SA increases NAc core, shell, and lateral DS MeCP2 expression in non-ELS rats but not in rats with a history ELS. Whereas, METH SA increased MeCP2 expression in the medial DS regardless of ELS experience. The transition from casual drug use to habitual drug abuse entails a gradual progression from initial NAc-dependent mechanisms to later DS-dependent mechanisms (Everitt & Robbins, 2013). Our observed interactions between ELS and METH SA on MeCP2 expression in these regions suggest that genetic regulation from MeCP2 may differentially influence stages of addiction development and maintenance.

Our findings are in agreement with the existing literature that exposure to psychostimulants increases MeCP2 expression throughout the striatum (Cassel et al., 2004; Im et al., 2010; Host et al., 2011), while providing new insights suggesting that the reward circuitry may have differing molecular responses to psychostimulants dependent on early life experiences. We previously reported that our ELS group self-administered more METH than the control handled group and had less MeCP2 expression in the NAc core (Lewis et al., 2013). It is possible that the decrease in MeCP2 expression was due to METH withdrawal, as brain tissue was collected more than two weeks following the last

METH exposure. This interpretation is in agreement with other findings that METH withdrawal produces a change in a multitude of transcription factors (Cadet et al., 2014).

The present study provides evidence that virally mediated MeCP2 knockdown in the NAc core influences both METH and sacc intake behavior. Interestingly, we found that while intake behavior for METH was affected immediately, group divergence in sacc SA did not take place until nine days after initiation. Additionally, while MeCP2 knockdown decreased motivation for METH in the progressive ratio paradigm it did not affect motivation for sacc. This suggests that NAc core MeCP2 expression mediates gene expression involved in motivation towards METH but not the non-drug reward sacc. MeCP2 knockdown did not affect either METH-primed drug seeking behavior or cue-induced drug seeking behavior for sacc. These results suggest that MeCP2 genetic regulation in the NAc core, while affecting METH and sacc intake, does not affect reinforcement seeking behavior, as measured by reinstatement. Interestingly, this mirrors our previous findings (Chapter 2; Lewis et al., 2013) that ELS influences METH SA but not cue-induced reinstatement. These results highlight that NAc core MeCP2 expression differentially mediates METH intake behavior, motivation, and drug-primed seeking in male rats. Importantly, our data also demonstrates that NAc core MeCP2 affects the reinforcing properties of METH and a non-drug reward differently.

Future research is needed to expand on these findings, such as investigation into the genes regulated by MeCP2 in brain reward regions after ELS and METH SA. Additionally, our findings did not elucidate specific cell types involved in these processes. This is an important distinction as both the NAc and DS have heterogeneous cell populations that contribute to different aspects of learning, motor control, and

reinforcement (Crittenden & Graybiel, 2011). Distinct cellular populations differ in genetic profiles, afferent and efferent projections, and contributions to various disorders (Crittenden & Graybiel, 2011). Therefore future research is required to determine if ELS and METH SA regulate MeCP2 expression in different populations such as the striosome and matrix departments and D1 and D2 receptor containing striatal neurons. Moreover, it remains to be determined if MeCP2 expression is regulating METH reinforcement through D1 and/or D2 containing NAc neurons. This specificity is important, as distinct and opposing roles for these neuronal subpopulations has been demonstrated in drug reward through interactions with BDNF (Lobo et al., 2010).

ELS has a well-established role in the etiology of many psychiatric conditions. Specifically, ELS alters the HPA stress response and dysregulation of this neuroendocrine system is implicated in disorders such as schizophrenia, post-traumatic stress disorder, major depression, and SUDs. An abundant amount of rodent research over the last decade demonstrated that ELS programs the adult HPA stress response through epigenetic regulation of various HPA genes including the genes coding for the GR, Avp, and POMC (Murgratroyd et al., 2009; Wang et al. 2014; Weaver et al., 2004). Additionally, post-mortem studies evaluating human brain tissue has also found a relationship between ELS and GR gene methylation and expression (Labonte et al., 2012; McGowan et al., 2009). Interestingly, the relationship between ELS and gene methylation status can also be evaluated in human peripheral tissue samples such as blood and buccal cells (Essex et al., 2013; Labonte et al., 2012; C Murgatroyd et al., 2015; Ouellet-Morin et al., 2012; Romens et al., 2014). While HPA dysregulation is involved in drug reinforcement and reward (Kosten & Ambrosio, 2002), an intriguing question remains -

can ELS also prime addiction vulnerability through epigenetic regulation in brain reward circuitry? Tesone-Coelho et al. (2013) demonstrated that ELS produced long lasting increases in MeCP2 expression throughout the striatum that may be responsible for greater opiate vulnerability in adulthood. Our data are in agreement with these findings, we also found ELS induced increases in MeCP2 expression in the NAc core and this epigenetic regulator may be responsible for increased vulnerability towards METH addictive behaviors. These findings implicate MeCP2 expression in the NAc core as a possible neuroepigenetic mechanism by which ELS may predispose individuals to respond differently to drugs of abuse. Our results highlight that experiences early in life can have long lasting effects on epigenetic regulation in reward circuitry that may shape vulnerability to METH abuse in adulthood.

CHAPTER 4

EARLY LIFE STRESS AND CHRONIC VARIABLE STRESS IN ADULTHOOD INTERACT TO INFLUENCE METHAMPHETAMINE SELF- ADMINISTRATION IN MALE RATS

Chronic METH abuse has dire effects on both society and users' health. METH is a highly addictive psychostimulant with worldwide abuse rates comparable to both heroin and cocaine combined (United Nations Office on Drugs and Crime, 2007). Therefore, identifying risk factors that contribute to the development of METH addiction is critical. ELS is implicated in long-term alterations in numerous behavioral and neurobiological systems that contribute to addiction vulnerability. The MS paradigm is a rodent model of ELS that has been shown to increase the rewarding and reinforcing properties of various drugs of abuse in adulthood (Moffett et al., 2007). While interactions between early life and adult stress differently modulate various neural systems and vulnerability to psychiatric illnesses such as schizophrenia and post-traumatic stress disorder (Choy et al., 2009; Yehuda et al., 2010), investigations into the effects of ELS in conjunction with adult stress on vulnerability to addiction-related behaviors has largely been unexplored.

The majority of research investigating the effects of ELS on addiction-like behaviors typically includes a single developmental insult. While this design allows for the detailed examination of effects of adverse early life events alone, it does not take into account the fact that many individuals with a history of ELS are also exposed to repeated or chronic stress later in adulthood. The purpose of the present study was to evaluate the effects of ELS, adult chronic variable stress, and their potential interactions on vulnerability towards METH addiction-related behaviors. Therefore, we utilized an

established paradigm of ELS (the MS paradigm) in conjunction with adult stress in the form of chronic variable stress. We hypothesized the combination of two developmental stressors would increase METH self-administration, delay extinction, and increase the magnitude of cue induced reinstatement compared to rats that either experienced only early life or adult chronic variable stress, or rats with no history of prior stress exposure.

Abbreviated Methods

Pregnant Long-Evans dams (Charles River Laboratory, Kingston, NY, USA) arrived on gestational day 12. On the day of birth (PND0), litters were assigned to one of two rearing conditions: maternal separation for 180 min/day (ELS) or 15 min/day (No Stress). The MS180 separation was chosen as a commonly utilized early life stressor, and the 15 min/day separation was chosen as control for handling that does not produce neurophysiological stress responses in the pups (Kuhn and Schanberg, 1998), and has previously been utilized for comparison to effects of prolonged separation (Ploj et al., 2003). Separation procedures were conducted on PND2-14. During PND15-20, litters were left undisturbed with the exception of once weekly cage cleaning. Litters were weaned on PND21, and female offspring were then removed from the study. Male offspring were sibling group housed until PND45 after which they were pair housed. On PND55, half the rats were exposed to a 10 day chronic variable stress paradigm (ELS+Adult Stress and Adult Stress groups). For 10 consecutive days, rats in the chronic variable stress condition received either two daily stressors or one daytime stressor and an overnight stressor (see Table 2 for details).

After chronic variable stress procedures concluded, all rats were implanted with intravenous catheters into the jugular vein using standard procedures as described

elsewhere (See Appendix). Rats were allowed five days of recovery prior to commencement of SA procedures. Rats were allowed to spontaneously acquire METH SA (0.05 mg/kg/infusion, delivered in a volume of 0.06 ml over a 2-sec period) by pressing a designated active lever in standard operant conditioning chambers (ENV-008; Med Associates, St. Albans, VT). An FR1 schedule of reinforcement was used in 2-hr daily sessions for 15 consecutive days. Each infusion was paired with a 2-sec auditory stimulus (~65 dB, 2900 Hz) and illumination of a stimulus light located above each response lever. Each drug infusion was followed by a 20-sec timeout period during which additional active lever presses were recorded but produced no programmed consequences. Next, all animals were subjected to extinction training, whereby presses on the active lever no longer produced any programmed consequences. Extinction training sessions were 2-hr in length and were conducted for eight consecutive days. On the day immediately following the last extinction session, all rats underwent cue-induced reinstatement testing (2-hr duration), whereby presses on the active lever resulted in the presentation of the tone and light previously presented during METH infusions, but did not result in drug infusion. One to two pups per litter were used for statistical analysis.

Results

Repeated measures ANOVA revealed a significant effect of time ($p = .003$) and a main effect of Early Life Stress on the number of METH infusions obtained per session, $F(1,23) = 13.00$, $p = 0.001$ (Figure 26), as well as the number of active lever presses per session, $F(1,23) = 14.73$, $p = 0.001$ (Figure 27). There was no main effect of Adult Stress on either the number of METH infusions or active lever presses, but there was a significant interaction between Adult Stress and Early Life Stress with regards to the

number of METH infusions obtained, $F(1,23) = 4.38, p = 0.04$, and active lever presses, $F(1,23) = 3.99, p = 0.04$. The effect of Adult Stress was dependent on Early Life Stress history, such that Adult Stress reduced responding for METH in the Early Life Stress group. No group differences in the number of inactive lever presses during self-administration were observed ($p > 0.05$, data not shown), nor were there any group differences in extinction rates or the number of active (Figure 28), or active lever presses during reinstatement testing (all p 's > 0.05 , Figure 29).

Discussion

The current study replicates our previous findings that ELS, induced by prolonged MS, increases adult METH intake compared to handled control rats (Chapter 2, 3; Lewis et al., 2013). Additionally, the current results also revealed that chronic variable stress in adulthood attenuates METH SA in rats with a history of ELS. However, adult chronic variable stress had no effect on METH SA in animals without a history of ELS. Contrary to our hypothesis, we found that animals exposed to both stressors self-administer less METH compared to animals exposed to ELS only. While the mechanism behind this behavioral difference is unclear, it is possible that these animals are more sensitive to METH and therefore self-administer less to obtain the same desired effect. Future studies assessing dose response curves are necessary to evaluate this possibility. Furthermore, it is possible that animals exposed to both early life and adult stress may enter a state of anhedonia, in which METH fails to produce reinforcing effects. It should be noted, however, that while inactive lever presses did not differ across treatment groups, additional studies are needed to confirm that reductions in general motor function did not influence these findings. Additional studies are needed to assess potential stressor

interaction in females, as well as in animals with no history of MS to assess potential effects of handling.

There are several possible mechanisms underlying the apparent protective effect of chronic variable stress against METH SA in male rats with a history of ELS. For example, the acute neuroendocrine stress response is considered a predictor for acquisition of psychostimulant self-administration, and adult chronic variable stress decreases the neuroendocrine stress response in rats with a history of ELS (Ladd et al., 2005; Renard et al., 2007). Adult CORT treatment following ELS desensitizes dopamine receptors without changing the receptor density in the NAc, a brain region associated with drug reinforcement (Choy et al., 2009). Additionally, chronic social defeat stress in adulthood increases thresholds for brain stimulation reward in rats with a history of ELS (Der-Avakian & Markou, 2010). Thus, our findings are in agreement with the extant literature suggesting that mild chronic adult stress can potentially reverse detrimental effects of ELS and modulate the reinforcing properties of psychostimulants. Overall, our results suggest that chronic variable stress alters the reinforcing effects of METH in animals with a history of ELS but does not alter extinction or cue-induced reinstatement of METH-seeking.

CHAPTER 5

THE EFFECT OF LITTER SEPARATION ON DAMS' CONDITIONED PLACE PREFERENCE TO METH

METH abuse continues to be a worldwide problem with use rates equivalent to heroin and cocaine combined (United Nations Office on Drugs and Crime, 2007). Intravenous METH use was first reported in 1959 and illicit production in California became rampant through the 1960's (Vearrier et al., 2012). As popularity and abuse rates grew the Comprehensive Drug Abuse Prevention and Control Act of 1970 placed amphetamines as Schedule II substances. Despite this, the drug remained popular and was associated with biker gangs throughout the 1970's and 1980's. During the 1980's and through the 1990's METH use spread throughout the West Coast and into the Midwest. For multiple decades METH use was predominated by Caucasian males in blue-collar careers (Vearrier et al., 2012). However, METH use among women has undergone a sharp increase the last few decades (Drug and Alcohol Services Information System [DASIS], 2008; Venios & Kelly, 2010).

The upward trend for METH abuse among women is concerning. METH usage is associated with increased energy, weight-loss, and intensifying sexual pleasure, all making it an alluring drug of choice for many women (Obert, 2005). Additionally, women have reported that METH induces a sense of power related to their sexual behavior that is rewarding (Lorvick et al., 2012). A recent review found that women are more susceptible to METH dependency and have earlier initiation to METH use than men (Dluzen and Lui, 2008). Women are now admitting themselves to rehabilitation with METH as their primary abused substance more than other abused substances. Whereas

men make up a larger proportion of abusers of heroin and cocaine, METH use is relatively equal among men and women (UCLA Integrated Substance Abuse Programs [UCLA ISAP], 2009).

The repercussions of drug abuse are paramount in female populations due to many factors. Indeed, SUDs in women have overlapping and unique characteristics in manifestation, attributes, and risks compared to men and the concerns are often more far-reaching (Brentari et al., 2011; Stocco et al., 2012). Inherently, women's drug use during pregnancy has negative implications for the infant. Prenatal exposure to METH leads to small for-gestational-age incidences and low birth weight, which are associated with negative health consequences including childhood mortality, morbidity, type 2 diabetes, and developmental delays (Smith et al., 2006). This is concerning as admissions for pregnant women using METH had a three-fold increase from 1994 to 2006 (Terplan et al., 2009). Additionally, women who used METH while pregnant were 12% more likely to develop a SUD than their non-pregnant METH using comparisons (Derauf et al., 2007). In a study done among mothers who use METH in the United States and New Zealand it was found that they had lower SES, delayed prenatal care, and increased rates of single parenting (Wouldes et al., 2013). SUDs typically impede parenting and mothers often struggle in recognizing and satisfying their children's needs (Barnard & McKeganey, 2004). Furthermore, mothers abusing drugs typically provide an at-risk environment for their children and the drug use has negative influences on the mother-child bond (Barnard & McKeganey, 2004; Stocco et al., 2012). Women with a SUD may struggle with feelings of guilt and failure pertaining to motherhood and their drug use, which leads to a feedback mechanism as increased drug use is used as a coping

mechanism. Unfortunately, these unique factors may contribute to women avoiding treatment.

Societal roles and support systems specific to women play a role in the development and recovery of SUDs (Tracy et al., 2010). For example, finding adequate childcare while in treatment may serve as a specific barrier to women. In 2013, less than 16% of the funds for Substance Abuse and Mental Health Services Administration were dedicated to women's specific needs. Furthermore, female representation is sorely lacking in biomedical research, and studies utilizing rodent models of addiction are not exempt from this criticism. In 2009, neuroscience research ranked last compared to other disciplines with 5.5 articles using only male subjects for every 1 article investigating females (Beery & Zucker, 2011). While there are practical reasons to investigate males alone, such as the 4-day estrous cycle affecting some experimental outcomes, it does not condone the lack of female specific testing. This bias in research results in findings, theories, ideas, and eventually treatments that are generalized to all, yet, only founded in data derived from male subjects. Understanding SUDs as they manifest differently in both sexes is necessary to develop effective treatments for SUDs. This is especially relevant given the established notions that all major classes of abused drugs interact with gonadal steroid hormones, and although males are more likely to use drugs of abuse, females tend to be more sensitive to them (Wood, 2010).

Sex differences in psychostimulant-induced behavioral effects in rats are well established. Specifically, female rats often display enhanced drug induced motor activity compared to males with cocaine and METH (Cailhol & Mormede 1999; Schindler et al., 2002). Additionally, a greater percentage of female rats acquire cocaine, heroin, and

METH SA (Carroll et al., 2001; Lynch & Carroll 1999; Roth & Carroll, 2004), and at a faster rate than male rats (Lynch & Carroll 1999). While work in female rodents utilizing models of addiction is of utmost necessity and merit, these studies are typically carried out in virgins or females who have never undergone pregnancy nor been exposed to pups. This distinction is of importance as pregnancy, lactation, and pup exposure affects monoamine, oxytocin, and GABAergic systems in various brain regions of the female rat brain (Lonstein et al., 2003; Lonstein et al., 2014; Macbeth et al., 2008; Rutherford et al., 2011), indicating the possibility that the rewarding and reinforcing properties of drugs may be altered during or after these experiences. Additionally, pup rearing behavior is regulated by brain structures overlapping with the reward network (hypothalamus, PFC, VTA, and NAc) (Rutherford et al., 2011), thus, these regions may respond to drugs differently after pup exposure. Therefore, to better model the population of women METH users who are post-childbirth, we investigated CPP to METH in dams immediately after their pups were weaned and virgin females. To model a stressful environment common to women with an SUD raising children, we separated the dams from their litters for 3 hours daily or 15 min daily during the first two weeks postpartum, an established stressor paradigm for postpartum rats (Banky et al., 1994; Boccia et al., 2007; Eklund et al., 2009; von Poser Toigo et al., 2012). We hypothesized that both postpartum groups would show greater CPP to METH compared to virgins. Additionally, we predicted dams separated from their litters for prolonged periods would show greater CPP to METH and greater CORT response to stress, than dams separated for short periods.

Abbreviated Methods

Animals, Litter Separation, and Stress Procedures

Pregnant dams and virgin females were purchased from Charles River Laboratories and arrived on gestational day 12 or ~PND85 (to correspond to the age of dams during CPP testing). Dams and virgins were housed individually in standard polycarbonate cages in a temperature and humidity controlled room with food and water available ad libitum. Litters were culled to a maximum size of 12 immediately upon discovery. Day of birth was considered postnatal day 0 (PND0). Dams were randomly assigned to one of two conditions: litter separation for 180 min per day (LS180) or 15 min per day (LS15). The separation procedure was conducted from PND2-14. At 8:00 am (reverse light cycle, lights off at 7:00 am) the dam was removed from the home cage and placed into a new cage with fresh bedding. The litters were then removed and placed into a separation cage kept in an isolated room. After the corresponding separation period, pups were returned to the home cage immediately prior to the dams return. During PND15-20 dams and litters were left undisturbed with the exception of once a week cage cleaning. Litters were weaned on PND21 the following day, dams received one 30 min restraint stress session. Immediately after the restraint session a blood sample was collected by tail bleed to assay blood stress hormone levels stress reactivity.

Methamphetamine Conditioned Place Preference

The day following restraint stress and blood sample collection, the dams underwent CPP procedures for METH. Rats received one 30-min habituation session prior to pre-conditioning preference testing. After individual preferences were determined, rats began five days of conditioning (30 min per session). Each animal

received METH (0.5 mg/kg i.p.) 15-min prior to placement in the initially non-preferred compartment, or saline (i.p.) 15-min prior to placement in the initially preferred compartment. Following five sequential conditioning days, animals were tested for place preference by allowing free access to both conditioning compartments for 30-min (See Appendix D). CPP score was calculated by subtracting time spent (measured in seconds) in the METH-paired chamber pre-conditioning from time spent (measured in seconds) in the METH-paired chamber post-conditioning.

Corticosterone Assay

Plasma CORT levels were determined by a standard enzyme-linked immunosorbent assay (ELISA) kit (Catalog# ADI-900-97 Enzo Corticosterone ELISA) following manufacturer's protocol. Briefly, 10 μ l of 1:100 steroid displacement reagent (SDR) was added to 10 μ l of each sample. Samples were then vortexed and diluted with 380 μ l EIA assay buffer.

Results

Estrous Cycle

A subset of rats received vaginal smears in order to assess if the estrous cycle affected the conditioned reward of METH. To verify phase, vaginal smears were taken prior to behavioral testing and divided into either estrous or di-estrous phase based on microscope visual analysis of cells (Goldman et al., 2007; Mennenga et al., 2015). No group differences were found in neither percent time spent in METH chamber ($p > 0.05$) nor CPP score ($p > 0.05$).

CPP Score

CPP score was calculated by subtracting the number of seconds spent in the METH-paired chamber pre-conditioning from the number of seconds spent in the METH-paired chamber post-conditioning. A one-way ANOVA revealed a main effect of litter separation, LS15 displayed a larger METH CPP score compared to LS180 and Virgins, (LS15: $n = 8$, LS180: $n = 10$, and Virgins: $n = 7$), $F(2, 24) = 4.29$, $p = .027$. LSD post-hoc comparisons LS15 vs LS180 $p = .013$, LS15 vs Virgins $p = .028$, LS180 vs Virgins $p = ns$ (Figure 30). The groups did not differ in Saline CPP score, $F(2, 24) = .48$, $p = .625$ (Figure 31).

Locomotor Activity

Groups did not differ in either Saline or METH primed locomotor activity (p 's $>.05$). All groups increased locomotor activity in response to METH compared to saline (LS15 $p = .01$, LS180 $p = .01$, Virgins $p <.000$) (Figure 32). There were no group differences in locomotor activity change score (Meth-Saline) (p 's $>.05$). No groups changed in saline primed locomotor activity over time, $F(2,15) = 1.91$, $p = 0.19$ (Figure 31). All groups changed in METH primed locomotor activity over time, $F(2,15) = 15.68$, $p = 0.001$, though not different from each other, $F(1,15) = 0.66$, $p = 0.53$ (Figure 31).

Plasma Corticosterone Levels

We performed ELISA for CORT levels in serum collected immediately following 30-min of acute restraint stress. One rat per group was removed from the analysis as an outlier ($>\pm 1.5$ SD from the mean). A one-way ANOVA demonstrated that difference in serum CORT concentration between groups, (LS15: $n = 8$, LS180: $n = 7$, and Virgins: $n = 7$) $F(3, 19) = 3.32$, $p = .05$ (Table 3). LSD *post-hoc* analyses revealed a significant CORT

elevation in LS15 rats compared to Virgins, $p = .02$, a trend-level difference between LS180 and Virgins, $p = .08$, and no significant difference between LS15 and LS180, $p = .52$ (Table 3).

Discussion

The rise of METH use among women is concerning. While animal research demonstrates that female rats may have higher vulnerability towards METH abuse (Roth & Carroll, 2004), it is important to evaluate female responses to drugs of abuse across the lifespan. The brain undergoes vast alterations in response to pregnancy, lactation, and pup rearing that may influence the behavioral and physiological response to drugs (Frankfurt et al., 2011; Seip et al., 2008). Additionally, we argue the importance of investigating maternal stress during the postpartum experience as this is likely most translationally relevant for women struggling with an SUD. In this study, we present evidence that dams exposed to short separations from their litters display higher levels of conditioned reward to METH, and trend towards higher stress induced CORT levels, compared to dams exposed to long separation and virgins. While we predicted postpartum females would be more sensitive to the rewarding properties METH compared to virgins, we also predicted that this effect would be exacerbated in the long litter separation group. Contrary to our prediction, our data suggests that the stress of the long separation negated the increased conditioned drug reward seen in postpartum females (Seip et al., 2008). While the mechanism behind the reduction in METH conditioned reward is unclear, it is possible that dams exposed to long litter separations have altered sensitivity to METH. If more sensitive, the METH dose may have produced aversive effects whereas, or if less sensitive, this dose may not have been sufficient to

produce CPP. Future studies assessing dose response curves are necessary to evaluate METH sensitivity in animals with this history of stressors. Furthermore, it is possible that dams exposed to long litter separations are in a state of anhedonia, in which METH does not produce rewarding effects.

Due to the established and robust relationship between ELS and adult psychiatric vulnerability, the vast majority of litter separation research has focused on the development of the offspring. However, litter separation also serves as a form of stress for the dam (Banky et al., 1994; Boccia et al., 2007; Eklund et al., 2009; von Poser Toigo et al., 2012). In humans, mothers separated from their children are more likely to have a SUD (Zlotnick et al., 2003). Therefore, animal models should be utilized to also investigate the effects of separation on the vulnerability to drug abuse in mothers. Moffett et al. (2006) did not find a difference in cocaine SA between dams exposed to litter separation and those not separated. Additionally, Kalinichev et al. (2000) found prolonged litter separations reduced the dam's sensitivity to morphine. To our knowledge we are the first to report on conditioned reward to METH in dams exposed to litter separation.

Recently, litter separation and has been shown to influence both anxiety and depressive-like behavioral phenotypes long after litter weaning (Boccia et al., 2007; Kalinichev et al., 2000; Maniam & Morris, 2010). Specifically, many have reported that long litter separation is associated with increased immobility behavior in the forced swim test, suggesting an increased depressive-like phenotype in the dam (Boccia et al., 2007; Maniam & Morris, 2010; Sung et al., 2010). The effects of long litter separation on behavior in the elevated plus maze is less consistent, some have reported increased

anxiety like behavior (Maniam & Morris, 2010), while others report less (Kalinichev et al., 2000). While we did not find a difference between groups in baseline locomotor activity or METH induced locomotor activity, we did not take any additional measures of anxiety or depressive like behaviors. It is apparent from these studies that litter separation has long lasting effects on anxiety and depressive-like behavior of the dam post-weaning and more investigations are warranted to further explore this relationship.

Litter separation not only influences dam behavior but also modulates various neurophysiological systems that may influence the rewarding and reinforcing properties of drugs of abuse. For example, long litter separation influences the dam's neuroendocrine system as it has been shown to increase plasma CORT levels, increase hypothalamic CRH mRNA, and decrease mRNA for glucocorticoid receptors in the hippocampus (Maniam & Morris, 2010). Here, we present data demonstrating all postpartum dams, exposed to either long or short forced litter separations, trended towards an increased CORT response to acute restraint stress. This is noteworthy as much research supports a link between the HPA axis and behavioral sensitivity to psychoactive drugs (Kosten & Ambrosio, 2002). Furthermore, serotonergic dysregulation has been implicated in addiction vulnerability and dams separated from their pups showed a decrease in expression of both serotonin and the rate limiting enzyme, tryptophan hydroxylase, in the dorsal raphe (Kirby et al., 2011; Sung et al., 2010). The GABAergic system plays a major role in the development of drug dependence and addiction (Addolorato et al., 2012) and during the postpartum experience the maternal brain goes through major alterations of this system (Lonstein et al., 2014). Interestingly, one prolonged litter separation has been shown to decrease GABA receptor expression,

highlighting the importance of investigating drug response in females after a stressful postpartum experience. Lastly, neurophysiological responses to catecholamines and an amphetamine-like drug can be measured by Na⁺/K⁺-ATPase activity (Rezin et al., 2013; Hernandez, 1992), and both long and short litter separation reduced Na⁺/K⁺-ATPase activity in various regions of the dam brain (von Poser Toigo et al., 2012). Clearly, stress in the form of forced litter separation alters various neurobiological systems that may influence the dam's response to drugs of abuse and thus, susceptibility towards addiction-like behaviors.

We demonstrate here that forced short separations from litters led to increased conditioned reward to METH and increased acute restraint induced CORT, at a trend level, in postpartum dams. In order to design adequate treatment strategies for women suffering from an SUD during the postpartum period, we must first understand the risk at a pre-clinical level. Therefore, there is a need to further this line of research, evaluating the effects of litter separation on the vulnerability to drug abuse in dams.

CHAPTER 6

GENERAL DISCUSSION

The collection of studies in this dissertation used three distinct paradigms in the rat to characterize how stress and epigenetics influence METH addiction-like behaviors. Our findings highlight that this relationship is not simple, but rather highly complex. We show that various types of stress exposure throughout development, and in both sexes, have multifaceted effects on METH intake, motivation, conditioned reward, and reinstatement. Moreover, for clinical translation, this work suggests that various events and experiences throughout the lifespan can influence behavioral response to drugs as well as the motivation to obtain them. We especially emphasize the robust influence of ELS on METH intake behavior, a relationship reflected in the clinical population.

Early Life Stress, MeCP2 Expression, and METH Addictive-Like Behavior

This series of experiments was the first to test the effects of ELS, in the form of prolonged maternal separation, on METH intake in male rats. ELS has known effects on the rewarding and reinforcing properties of other drugs of abuse including cocaine, amphetamine, and opiates (Moffet et al., 2006; Der-Avakian et al., 2010; Vazquez et al., 2005). In Chapter 2, we show that exposure to ELS has a robust effect on METH intake behavior in adult male rats. Furthermore, we found that ELS exposure and METH intake patterns were related to MeCP2 expression patterns in the NAc core. Next, in Chapter 3, we investigated the possible long-term effects of ELS and/or interactions with subsequent METH SA on MeCP2 expression in multiple reward-related brain regions. We found that ELS and METH SA have unique and interactive effects on MeCP2 expression throughout these brain reward regions. Specifically, ELS leads to a long lasting increase in MeCP2

expression in the NAc core and METH, self-administration increases MeCP2 expression in the striatum in an ELS dependent fashion. Further, we provide evidence that virally mediated reduction of MeCP2 expression in the NAc core decreases METH intake behavior. The current findings are in agreement with the literature demonstrating that ELS can lead to long lasting effects on the epigenome (Weaver et al., 2004; Tesone-Coelho et al., 2013), psychostimulant exposure increases MeCP2 expression (Cassel et al., 2004; Im et al., 2010; Host et al., 2011), and that striatal MeCP2 expression impacts the reinforcing and rewarding properties of psychostimulants (Deng et al., 2010; Im et al., 2010). Taken together, this collection of studies demonstrates that ELS may epigenetically prime individuals to be at greater risk for a SUD via lasting changes in the brain reward circuitry.

Early Life Stress, Adult Chronic Stress, and METH Intake Behavior

Interactions between ELS and adult stress differently modulate various neural systems and vulnerability to psychiatric illnesses such as schizophrenia and post-traumatic stress disorder (Choy et al., 2009; Ozer et al., 2008). Therefore, in Chapter 4, we investigated the combined impact of ELS and chronic variable stress in early adulthood on METH intake behavior. Firstly, we replicated our previous findings, as described in Chapter 2 and 3, that ELS induced by prolonged MS increases adult METH intake compared to handled control rats. Additionally, our data revealed that chronic variable stress in adulthood actually attenuates METH SA in rats with a history of ELS. However, adult chronic variable stress had no effect on METH self-administration in animals without a history of ELS. These findings were unexpected, since instead of demonstrating that the two types of stressors produced additive effects of increasing

METH SA, it appears that chronic variable stress in adulthood confers resiliency towards reduced METH intake. Our findings are in agreement with the extant literature suggesting that mild adult stress can potentially reverse detrimental effects of ELS and modulate the reinforcing properties of psychostimulants (Ladd et al., 2005; Renard et al., 2007; Choy et al., 2009; Der-Avakian & Markou, 2010). This study highlights the complex interplay between experiences throughout the lifespan and how they may interact to modulate the neurophysiological response to drugs and subsequent SUD vulnerability.

Postpartum Stress and Conditioned Reward to METH

The prevalence of METH abuse among women is on the rise and women who have their children taken from them are more likely to have an SUD. In Chapter 5, we argue for the need to evaluate this relation by utilizing the common maternal separation paradigm. While much research has investigated the effects of maternal separation on the adult offspring, few reports evaluate the effects on the dams, and even fewer on the dam's addiction vulnerability. Our study is the first to report on the effects of stress during the postpartum experience on post-weaning conditioned reward to METH in dams. In this study, we present evidence that dams exposed to short separations from their litters display higher levels of conditioned reward to METH compared to dams exposed to long separation and virgins. While we predicted postpartum females would be more sensitive to the rewarding properties METH compared to virgins, we also predicted that this effect would be exacerbated in the long litter separation group. Contrary to our prediction, our data suggest that the stress of the prolonged litter separation negated the increased reward often seen in postpartum females (Seip et al., 2008). Again, these data

demonstrate that the relationship between stress and METH reward and reinstatement is highly complex. Evidence shows that females have a different addiction like phenotype compared to males (Roth & Carroll, 2004) and that this phenotype can change throughout the postpartum experience compared to virgins (Seip et al., 2008). Additionally, accumulating evidence demonstrates that stress during the postpartum experience leads to alterations in the postpartum brain and behavior (Banky et al., 1994; Boccia et al., 2007; Eklund et al., 2009; von Poser Toigo et al., 2012). Therefore, more translationally relevant research is needed to model this unique period of vulnerability in women.

Final Conclusions

In summary, METH use disorder is a chronic neuropsychiatric illness characterized by complex clinical symptoms. It is important to note that human METH addiction manifests differently in the clinical population, with different intake patterns, amount of drug intake, and comorbidities such as depression, suicidal ideations, and psychotic symptoms (Cadet, 2014). Therefore, to accurately study the neural, (epi)genetic, and psychopharmacological aspects of addiction, we must employ a diversity of animal paradigms (Cadet, 2014). Large strides have been made during the last 50 years in our understanding of the actions of abused drugs in the brain. Yet, this information should not be confused as having a better understanding of addiction etiology. One of the most important questions in addiction research today, is why the vast majority of individuals are exposed to drugs of abuse in their life time yet only a small percentage develops a SUD (Le Moal, 2009).

The current prevailing notion is that SUDs develop as a consequence of chronic exposure to drugs. These drugs act on brain structures evolved for motivation, learning,

and reward. Thus, abused drugs mimic the effects of natural rewards and reinforcers to cause an artificial pleasure state and intense and uncontrolled drug craving (Vrecko, 2010), essentially ‘hijacking’ the reward circuitry (Leshner and Koob, 1999). Caution is warranted against the conceptualization and reduction of SUDs as primarily genetic, neurological, or biological disorders. This reasoning denies the importance of social context, such as developmental factors, in the initiation, use and misuse, cessation, abstinence, and relapse behaviors concerning drugs (Galea et al., 2004). The collection of studies described in this dissertation use three different rodent paradigms to model the complex interaction between social stressors and the reinforcing and rewarding effects of METH. The reported results underscore the importance of evaluating the past and current social environments in the etiology of SUDs and subsequent treatment plan for each individual patient. Furthermore, to staunchly promote addiction vulnerability as primarily biological allows the general public and policy makers to all but ignore the massive amount of evidence suggesting SES and other social factors play a major role in all stages of SUDs (Galea et al., 2004). As addiction researchers, we must recognize the inherent dilemma in our current drug policy. The National Institute of Health’s division of Drug Abuse is the primary funding agency for research yet, the “War on Drugs” and criminalizing of addicts is still prevalent. Finally, SUDs are a substantial socioeconomic and psychiatric concern for modern society, therefore, it is necessary to re-examine our failing controlled substance policies. For example, as opposed to treating drug use as a criminal act, there are potential beneficial effects for decriminalization of drug use in order for addicts to receive medical care through individualized treatment plans. Finally,

re-examining our controlled substance policies may open for the door for research into novel addiction treatments, such as psychedelic assisted cognitive behavioral therapy.

Research in the 21st century has thus far demonstrated that exogenous and endogenous factors contributing to behavior and psychiatric vulnerability are intimately linked. For example, gene by environment studies demonstrate an interaction in which our environment shapes us and we shape our environment. The field of neuroepigenetics shows that our environment influences genetic expression that determines behavior. As discussed by Dr. Nancy Eisenberg, in a presidential column of the Association for Psychological Science journal, the modern field of psychology is at risk of jeopardizing our understanding of human functioning through reductionist thinking (Eisenberg, 2014). Therefore, while modern neuroscience techniques evolve and allow us to answer novel and important molecular questions; we must not lose sight of the human condition as dynamic, with various levels of complexity that cannot be fully measured at any single level.

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APPENDIX A

ANIMALS AND MATERNAL SEPARATION PROCEDURES

All experimental and surgical procedures were carried out in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee of Arizona State University.

Pregnant dams were purchased from Charles River Laboratories and arrived on gestational day 12. Dams were housed individually in standard polycarbonate cages in a temperature and humidity controlled room with food and water available ad libitum. Beginning on gestational day 20 (range of gestation 21-23 days) cages were checked for delivery of pups three times a day. Litters were culled to a maximum size of 12 immediately after discovery. Litter sizes ranged from 10-12 with one litter at 8 due to pup attrition. The litter sex ratios were left natural. Day of birth was considered postnatal day 0 (PND0).

Litters were randomly assigned to one of two conditions: MS for 180-min per day (MS180 or Early Life Stress) or the handled control group, 15-min per day (MS15, No Stress, or Handled Control). The separation procedure began on PND2. At 8:00 am (reverse light cycle, lights off at 7:00 am) the dam was removed from the home cage and placed into a new cage with fresh bedding. The pups were then removed and placed into a separation cage kept in an isolated room. Heat lamps were set over the separation cages and maintained at $30 \pm 0.5^\circ \text{C}$ to $32 \pm 0.5^\circ \text{C}$ to prevent hypothermia. The pups were left unattended during the corresponding separation period then returned to the home cage immediately prior to the dams return.

After separation procedures, rats were left undisturbed with the exception of once a week cage cleaning performed by Department of Animal Care and Technologies

(DACT) employees. Pups were weaned on PND21 into same sex group housing, and pair housed with a sibling on PND40-45. Females were not used for the remainder of the any study.

APPENDIX B

BILATERAL INTRACEREBRAL VIRUS INJECTION

Rats were anesthetized with isoflurane (2% v/v) vaporized in oxygen at a flow rate of 2 L/min. Rats were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). The scalp was shaved and surgical area cleaned with ethanol and 1% iodine before a ~2 cm incision was made to expose the skull. Guide cannulae (26G, Plastics One, Roanoke, VA) were aimed bilaterally into the NAc core. The following stereotaxic coordinates were used (in mm from bregma and skull surface): anterior +2.5, lateral \pm 1.5, from skull surface -6.0. An adeno-associated virus (AAV) expressing a short hairpin loop RNA (shRNA) against rat MeCP2 mRNA (sh-MeCP2), or an empty control virus (sh-Control), was synthesized by Virovek Inc (Hayward, CA) and bilaterally infused in a volume of 2 μ l per side (2.25×10^{13} particles/ml). Viruses were delivered over a 5 min period, after which the injector was left in place for an additional 10-min to allow for virus diffusion. Following withdrawal of the injector, skull holes were filled with bone wax, and the scalp sutured closed before treatment with topical antibiotic and analgesic ointments. Following surgery, animals remained single-housed in their home cage for two weeks prior to behavioral testing.

APPENDIX C
CHRONIC VARIABLE STRESS EXPOSURE

For data collected in Chapter 5, at PND55 half of the rats were exposed to 10 consecutive days of chronic variable stress in which they either received two randomly assigned daily stressors or one daytime stressor and one overnight stressor. Day time stressors consisted of: 1) loud white noise (~100 dB) for 2-hr,-2) tail pinch with a clothespin during restraint for 10 min, 3) restraint in a Plexiglas rodent restrainer for 2-hr,-4) forced swim in a cylinder container (45 X 20 X 30 cm) in room temperature water for 10-min,-5) strobe light exposure for 2-hr,-6) placement of the cage on an orbital shaker for 2-hr, and -7) subcutaneous saline injections (1 ml/kg). Overnight stressors consisted of placing another rat's dirty bedding in the home cage and overcrowding (maximum of 8 rats per cage).

APPENDIX D
CONDITIONED PLACE PREFERENCE

An unbiased apparatus with two distinct conditioning compartments was used. Walls of each compartment were visually distinct. In addition, the floor texture was made different in each compartment, with a smooth floor on one side and wire mesh or material on the other side. Rats were habituated to the testing apparatus during a single 30-min session with free access to both conditioning compartments. Animals were then subjected to a 30-min preconditioning test to determine any initial preference for one of the conditioning compartments. Each animal received METH (0.5 mg/kg i.p.) 15 min prior to placement in the initially non-preferred compartment, or saline (i.p.) 15 min prior to placement in the initially preferred compartment. Next, animals underwent place conditioning in twice-daily 30-min conditioning sessions. Saline-conditioning sessions were conducted in the morning, and METH-conditioning sessions were conducted in the afternoon to avoid carryover effects. Following five sequential conditioning days, animals were tested for place preference by allowing free access to both conditioning compartments for 30-min. The dependent variable assessed was total CPP score, calculated by pre-conditioning time spent in the METH chamber subtracted from post-conditioning time spent in the METH chamber.

APPENDIX E
IMMUNOBLOT ANALYSIS

Dissected brain tissue (1 mm punch diameter) was placed in a neuronal protein extraction reagent (N-PER, G-Biosciences, USA) containing a protease inhibitor cocktail. Tissue was homogenized with a Branson sonicator and centrifuged at 10,000g at 4°C for 10 min. The supernatant was then stored at -80°C until analysis. Protein concentrations were determined using bicinchoninic acid (BCA) protein assays (Bio-Rad). For immunoblot analysis, samples (30 µg of protein per lane) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 4-12% RunBlue gels (Expedeon). Proteins were transferred to nitrocellulose membranes, pre-blocked with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat dried milk powder for 1-hr before overnight incubation with primary antibodies polyclonal antibody: rabbit anti-MeCP2 (Abcam; 1:100 dilution) and as a loading control, rabbit anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam; 1:10000 dilution). Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antisera (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:1500 dilution) for 1-hr, and immunoreactive bands were detected by enhanced chemilluminescence (Western Lightning Plus) and exposure to Kodak Bio-Max x-ray films. Developed films were then scanned into a computer for analysis using ImageJ Tool software package. For each sample, the optical density of the MeCP2 band was divided by that of the corresponding GAPDH band to yield a MeCP2/total GAPDH ratio.

APPENDIX F
IMMUNOHISTOCHEMISTRY

Brain tissues were collected on the day following behavioral testing. Rats were deeply anesthetized with either 150 mg/kg i.p. sodium pentobarbital, or exposure to isoflurane, and perfused transcardially with ice-cold 0.1 M PBS followed by ice-cold 4% w/v paraformaldehyde (PFA) in PBS, pH 7.4. Brains were removed, post-fixed in 4% PFA overnight and stored in 30% w/v sucrose in PBS. Brains were sectioned (35 μ m thickness) in the coronal plane on a cryostat (Leica CM1900, Bannockburn, IL). Sections were then rinsed 3 x 10 min in PBS containing 0.1% v/v Tween 20 (PBST) followed by incubation in PBST containing 5% v/v normal donkey serum for 1-hr. Sections were then incubated overnight under gentle agitation at 4°C in PBST containing a rabbit anti-methyl CpG binding protein 2 polyclonal antibody (1:200 dilution; Thermo Scientific) and then rinsed 3 x 10 min in PBS. Sections were then incubated in PBS containing AlexaFluor 488 conjugated donkey anti-rabbit IgG antisera (1:200; Jackson ImmunoResearch, West Grove, PA) and then rinsed 3 x 10 min in PBS. Sections were mounted on microscope slides using VectaShield mounting media (Vector Labs, Burlingame, CA), coverslipped, and stored in darkness until imaging.

Investigator was blind to treatment condition during microscopic analysis. Sections were visualized at 200x magnification. Digital images of the selected area were obtained and counted for MeCP2 immunoreactivity by observers blind to treatment conditions using the ImageJ Tool software package (National Institutes of Health, Bethesda, MD). A total of six sample areas were counted for each subject (i.e., 1 sample area/2 hemispheres/3 sections). Care was taken to ensure that the sections for each subject that were labeled came from the same anatomical level within each plane. The counts from all six sample areas from a particular region were averaged to provide a

mean number of immunoreactive cells per animal to be used as an $n = 1$ for statistical analysis (Thiel et al., 2010).

APPENDIX G

JUGULAR VEIN CATHETER SURGICAL PROCEDURES

Rats were implanted with intravenous catheters into the jugular vein on PND60 (\pm 1). Rats were anesthetized with isoflurane (2% v/v, Butler Schein Animal Health, Dublin, OH) vaporized in oxygen at a flow rate of 2 L/min. Rats received pre-incision injections of buprenorphine (0.05 mg/kg, s.c., Reckitt Benckiser, Richmond, VA) and meloxicam (1 mg/kg, s.c., Boehringer Ingelheim, St. Joseph, MO). Surgical sites were shaved and cleaned with 1% iodine. A ~2 cm incision was made in order to isolate the right or left jugular vein. A sterile silastic catheter filled with heparinized saline and inserted 2.5 cm into the vein. The catheter was secured to the surrounding tissue with sutures, and the opposite end of the catheter was tunneled subcutaneously to the dorsum where it exited the skin between the scapulae. A mesh collar attached to a threaded vascular access port (Plastics One, Roanoke, VA, USA). The wound was then treated with 0.2 ml bupivacaine hydrochloride (0.25% v/v), closed with nylon sutures (Ethicon, San Lorenzo, Puerto Rico) and topically treated with topical lidocaine and a triple antibiotic gel. The access port was sealed with a piece of Tygon tubing closed at one end and a threaded protective cap (Plastics One). Rats were given small portions of sweetened cereal to facilitate postsurgical rehabilitation. Following surgical procedures, rats were allowed 5-7 days minimum of recovery and received daily intravenous infusions of 0.2 ml Timentin in sterile heparinized saline to minimize infections and maintain catheter patency.

APPENDIX H
RESTRAINT STRESS AND BLOOD COLLECTION

Dams were placed into cylindrical plastic restraint tubes (8.5 cm diameter x 21.6 cm length) once for 30 min prior to blood sample collection to be assayed for corticosterone levels. Blood samples were obtained from the tail using a 26 gage needle to draw 3-4 drops of blood into heparinized blood collection tubes. Blood samples were immediately centrifuged for 15 min at 12000 rpm at 4°C. Separated plasma was immediately aspirated into new centrifuge tubes and stored at -80°C prior to corticosterone assessment.

APPENDIX I
ROTATIONAL MOTOR ACTIVITY

The Rotorat apparatus consisted of a stainless steel bowl (40.6 cm diameter x 25.4 cm height; model ENV-500, Med Associates, St. Albans, VT) surrounded by clear acrylic walls. A spring tether attached to a rotational sensor was suspended from the center of the apparatus to record rotational motor activity. A zip-tie collar was loosely placed around the neck of the rat and connected to the spring tether via a stainless steel alligator clip to record 2, 180, and 360 degree movements for 30 consecutive min.

APPENDIX J

SELF-ADMINISTRATION APPARATUS

Behavioral testing was conducted in SA chambers (ENV-008; Med Associates Inc., St. Albans, VT) that were interfaced to a PC computer and located in sound attenuating melamine enclosures equipped with ventilation fans. The chambers (28 x 27 x 22 cm) consisted of two aluminum walls and two clear Plexiglas walls. The ceiling was also constructed of Plexiglas with a 3 cm diameter hole cut in the center to allow a drug delivery tether to pass through. The floor consisted parallel stainless steel rods (0.48 cm diameter) placed 1.6 cm apart. Each chamber contained a house light located 1.25 cm from the ceiling, a Sonalert speaker that provided an auditory stimulus (~65 dB, 2900 Hz) during drug infusion, one retractable response lever, one stationary response lever, and two 2.5 cm stimulus cue lights located above each response lever. The retractable lever was designated the active lever as an additional cue for drug availability. Response levers were located 7 cm above the floor of the chamber. For the data collected in Chapter 2 and 3, levers were replaced with nose-poke operant response in the same position as the levers. Centered between the levers was a 5 x 5 cm food pellet receptacle. Each chamber was outfitted with a single-speed automated drug infusion pump (PHM-100; Med Associates). Tygon microbore tubing (0.5 mm ID) was used to connect the syringe containing the drug solution to a single-channel liquid swivel that was mounted to the top of the chamber enclosure. The swivel was then connected to the vascular access port using Tygon microbore tubing that was protected by a stainless steel tether (Plastics One, Roanoke, VA). All experimental parameters were controlled using Med PC IV software (Med Associates).

APPENDIX K
SELF-ADMINISTRATION, EXTINCTION,
PROGRESSIVE RATIO,
AND CUE- AND DRUG-INDUCED REINSTATEMENT

For data collected in Chapter 2, 3 (study 1), and 4, male rats underwent 2-hr daily SA sessions whereby active operant responses resulted in delivery of METH (0.05 mg/kg per infusion, delivered in a volume of 0.06 ml over a 2-sec period) on an FR1 schedule of reinforcement. For data collected in Chapter 3 (study 2), male rats underwent 6 hr daily SA sessions whereby active operant responses resulted in delivery of METH (0.05 mg/kg per infusion, delivered in a volume of 0.06 ml over a 2-sec period) on a FR1 schedule of reinforcement. Rats progressed to FR3 and FR5 schedules of reinforcement after earning at least 10 infusions on two consecutive days. Each METH infusion delivery was followed by a 20-sec timeout period, during which additional active responses were recorded but produced no drug infusions. Each infusion was accompanied by concurrent illumination of a stimulus light located directly above the active lever/nose-poke, and presentation of an auditory stimulus for 2-sec. SA sessions were conducted 7 days per week. METH hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% sterile saline.

For data collected in Chapters 2 and 4, after METH self-administration procedures ended all animals were subjected to extinction training, whereby active responses no longer produced any programmed consequences (i.e., no tone/light presentation and no activation of the syringe pump). Extinction training sessions were 2 hr in length. For data collected in Chapter 2, 3 (sacc trained animals), and 4 on the day immediately following the last extinction session, all rats underwent cue-induced reinstatement, whereby active responses produced the tone and light cue previously presented with a reinforcement, but did not deliver a reinforcer. For data collected in Chapter 3, on the 2 days following self-administration all rats were tested on the

progressive ratio paradigm, in which the number of lever presses required to obtain an infusion of METH was determined by the following equation: responses per reinforcer delivery = $5 \times e^{(\text{injection number} \times 0.2)} - 5$ (i.e., 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, etc) (Richardson and Roberts, 1996). After completion of progressive ratio testing, rats self-administered METH on an FR1 schedule of reinforcement for two days before starting extinction training. On the day following the last extinction training session, rats were subjected to drug-primed reinstatement in which they received a 1mg/kg i.p. injection of METH 30 min prior to placement in operant chambers.

APPENDIX L
SH-RNA VIRUS DESIGN

A shRNA directed against rat MeCP2 mRNA (Mecp2-shRNA, target sequence ctaaagtag, start position 450, sense strand 5'-tgccttcgctctaaagtagttcaagagactactttaga-gcgaaaggctttttc-3, antisense strand 5'-tcgagaaaaagccttcgctctaaagtagtctctgaactactttagagcg-aaaggca-3'), was synthesized by Virovek (Hayward, CA) as previously described and optimized by Jin et al. (2008). The shRNA was incorporated into vector containing the U6 pol III and cytomegalovirus (CMV) promoters along the coding sequence for green fluorescent protein (GFP), and packaged into an AAV (serotype 9, 2.25×10^{13} particles/ml). The resulting viral vector (AAV9-U6-Mecp2-shRNA-CMV-GFP) (sh-MeCP2) was suspended in PBS containing 0.001% pluronic F-68 and filter sterilized. An empty virus lacking the shRNA sequence (AAV9-U6-CMV-GFP, 2.25×10^{13} particles/ml; sh-Control) served as a control.

APPENDIX M
TRANSCARDIAL PERFUSION

Animals were anesthetized using isoflurane. Once the animal became unresponsive to the toe pinch-test, a 5-6 cm lateral incision was made through the abdominal wall just beneath the rib cage. Care was taken to separate the liver from the diaphragm, then a cut through the diaphragm was done to expose the pleural cavity. A similar cut was made on the contralateral side. Next, a 15-gauge olive-tipped perfusion needle was used to puncture through the heart and enter the ascending aorta. The needle was then secured using a hemostat to clamp the heart. Finally, the animal's right atrium was cut. The animal was then perfused first with ice cold phosphate-buffered saline (PBS) and then ice-cold 4% paraformaldehyde. The brain was then quickly removed and stored in 4% PFA in -20°C before transferring to a 30% sucrose solution and stored in -20°C prior to cryosectioning at 35 μm thickness using a Leica CM1900 cryostat.

Table 1

Mean ± SEM Lever Presses Across 15 Sessions of METH SA

Mean ± SEM Across 15 Sessions of METH SA		
	<u>MS15</u>	<u>MS180</u>
<hr/> Self-Administration <hr/>		
Active Lever Presses	175±22	446±108
Inactive Lever Presses	394±88	362±52
<hr/> Extinction <hr/>		
Active Lever Presses	147±22	151±10
Inactive Lever Presses	302±100	117±16
<hr/> Reinstatement <hr/>		
Active Lever Presses	18±4	24±3
Inactive Lever Presses	7±3	8±2

Table 2

Example Chronic Variable Stress Procedure

Example Chronic Variable Stress Procedure						
Day	First Stressor	Duration	Second Stressor	Duration	Overnight stressor	
1	Loud noise	2hr	Tail-pinch	10min	X	
2	Forced swim	10min	X		Dirty bedding	
3	Restraint	2hr	Strobe	2hr	X	
4	Tail-pinch	10min	X		Crowding	
5	Shaker	2hr	SC Injection	~2min	X	
6	Loud noise	2hr	Tail-pinch	10min	X	
7	Forced swim	10min	X		Crowding	
8	Restraint	2hr	Strobe	2hr	X	
9	Tail-pinch	10min	X		Dirty bedding	
10	SC Injection	~2min	Shaker	2hr	X	

Table 3

Effect of Acute Restraint Stress on Plasma CORT Levels

Effect of Acute Restraint Stress on Plasma CORT Levels (ng/ml)			
Group	LS15	LS180	Virgins
Mean (\pmSD)	147 (\pm 35)	134 (\pm 48)	95 (\pm 36)

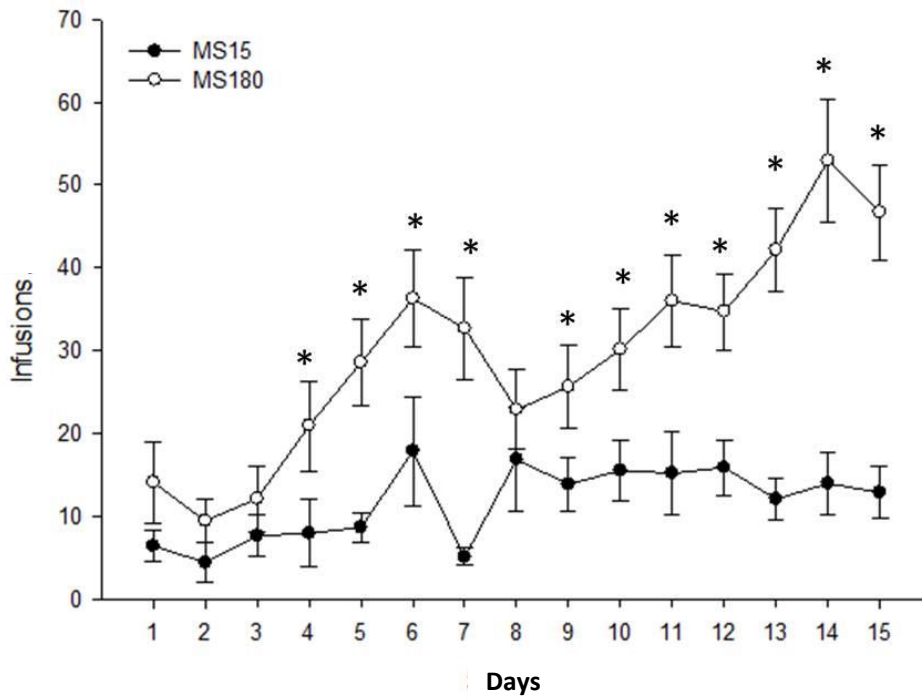


Figure 1. MS180 rats earned more METH infusions (0.05 mg/kg) in 15 daily 2 hr sessions compared to MS15 rats. MS15 (n = 9) and MS180 (n = 17) rats. Data represent mean ± SEM. * $p < 0.05$ vs. MS15.

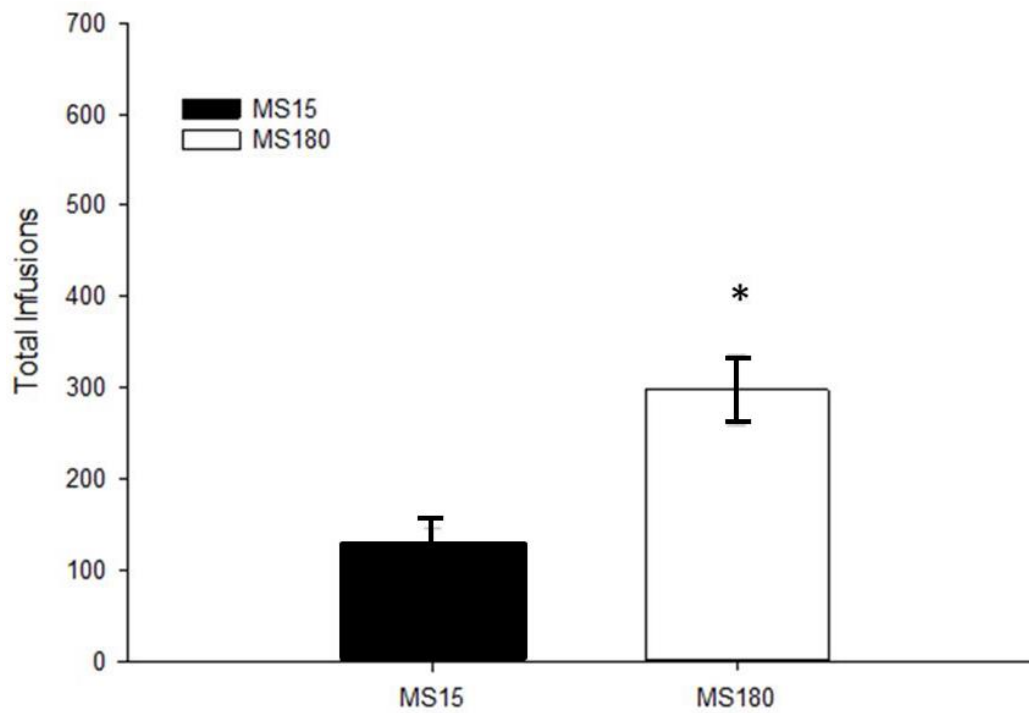


Figure 2. MS180 rats earned more total METH infusions compared to MS15 rats. Total number of METH infusions earned across 15 daily 2-hr sessions in MS15 (n = 9) and MS180 (n = 17) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. MS15.

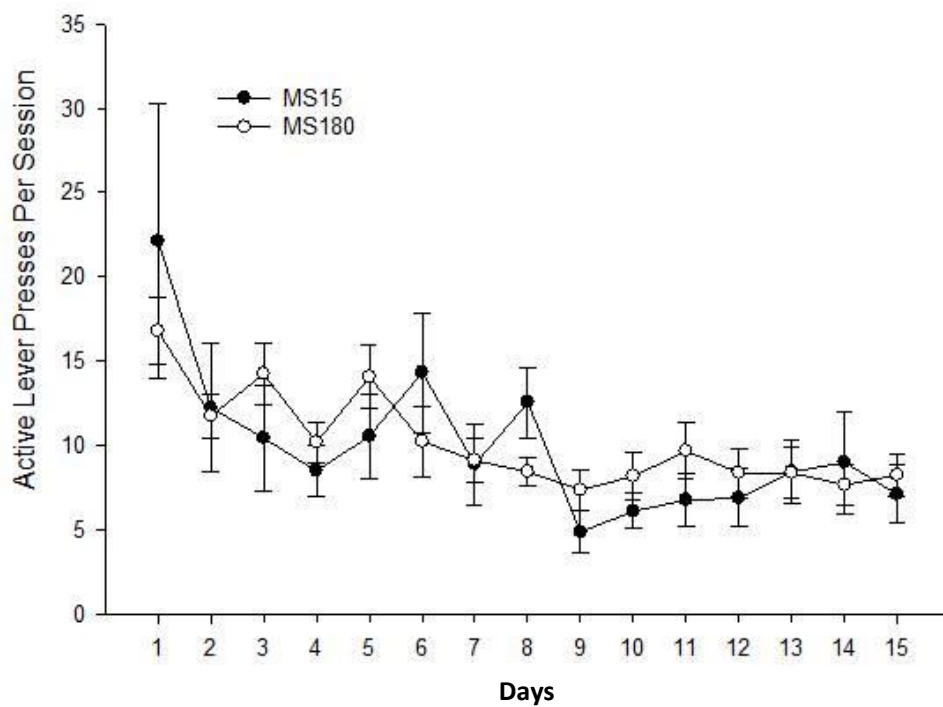


Figure 3. We observed no group differences in active lever presses during extinction training. Average number of active lever presses per 2-hr session for 15 consecutive days during extinction in MS15 (n = 9) and MS180 (n = 17) rats. Data points represent group mean \pm SEM.

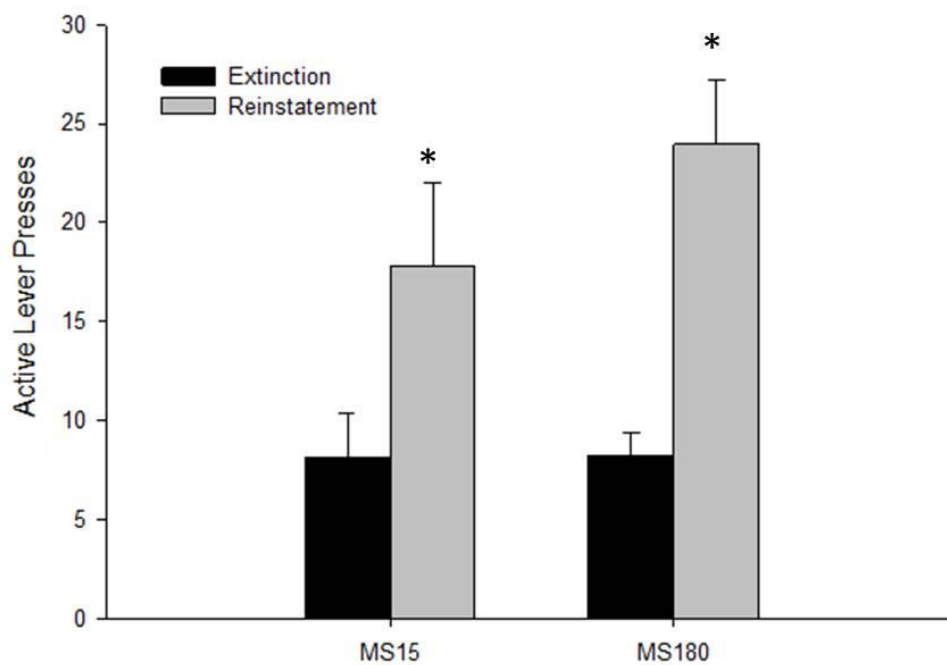


Figure 4. We observed no group differences in active lever presses during cue-induced reinstatement testing. Number of active lever presses across the final two days of extinction training (black bars) and during cue-induced reinstatement (grey bars) in MS15 (n = 9) and MS180 (n = 17) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. extinction.

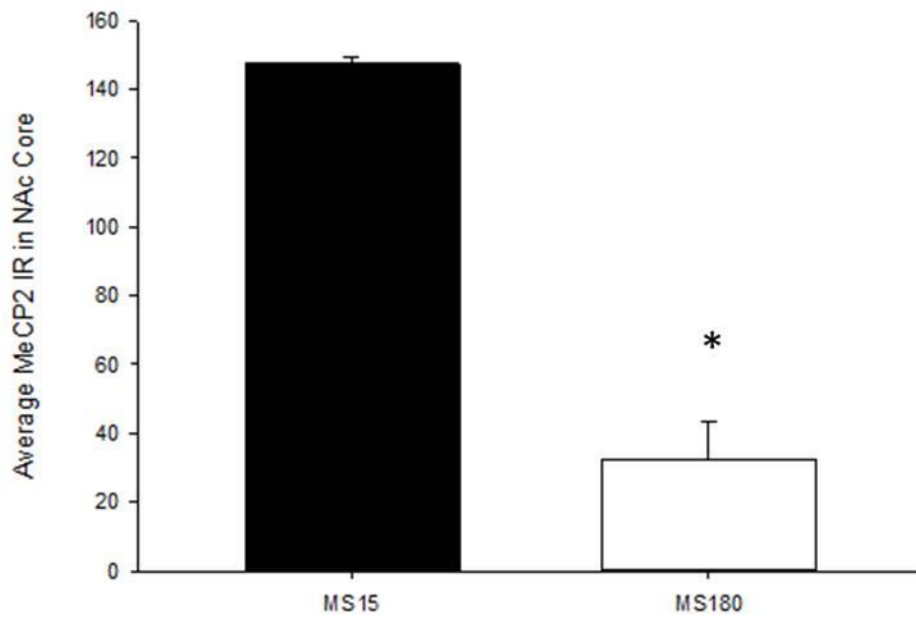


Figure 5. MS15 rats (black bar) exhibited more MeCP2 immunoreactivity in the NAc core compared to MS180 rats (white bar). Cell counts for MeCP2 immunoreactivity in the NAc core for MS15 (n = 5) and MS180 (n = 5) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. MS15.

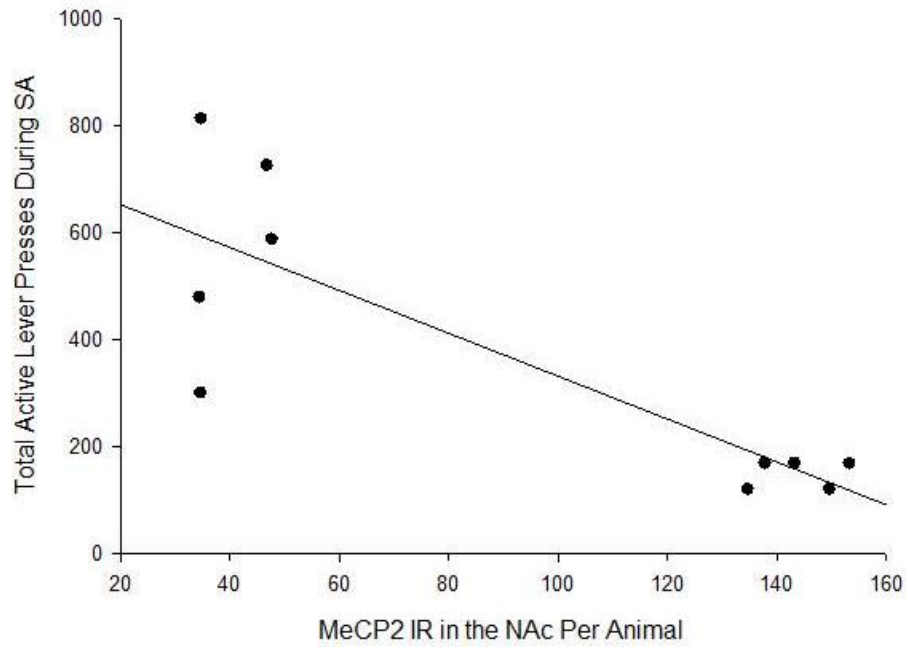


Figure 6. The total number of active lever presses over 15 days of SA was negatively correlated with MeCP2 immunoreactivity in the NAc core ($r = -0.836$, $p = 0.003$).

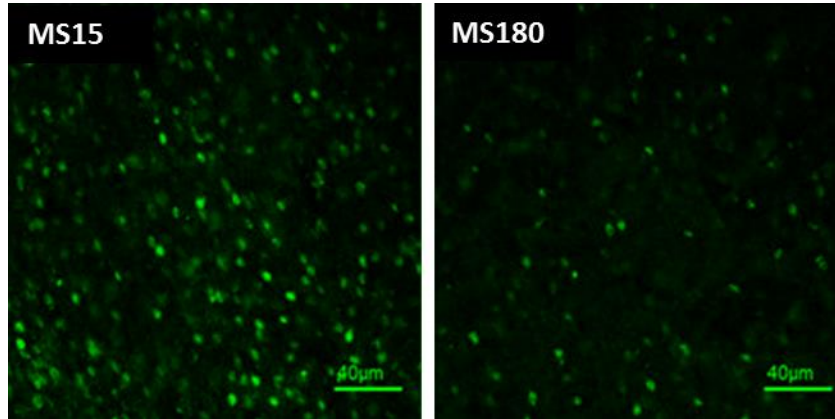


Figure 7. Representative photomicrographs of MeCP2 immunolabeling in the NAc core of MS15 and MS180 rats. NAc core was determined using the anterior commissure as a landmark.

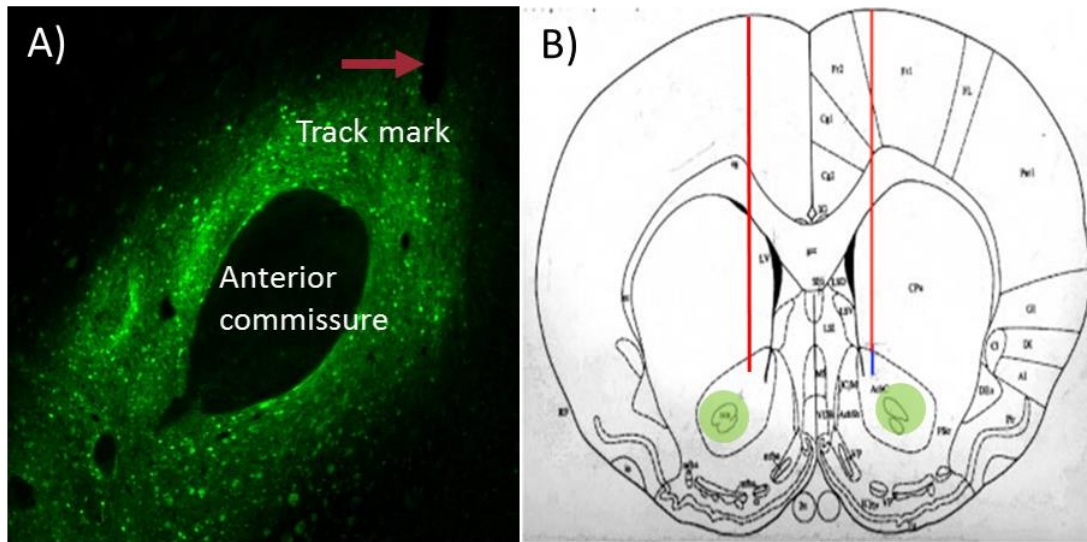


Figure 8. A) Representative photomicrograph of the virus reporter gene GFP and track mark in the NAc core of a single male rat. Scale bar 130 μm . B) Brain atlas schematic of viral injection sites in the NAc core.

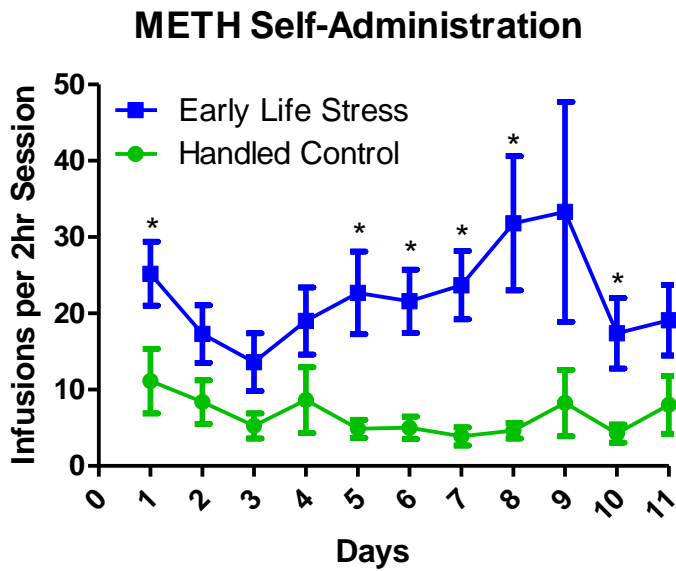


Figure 9. The Early Life Stress group earned more infusions compared to the Handled Control group. Average number of infusions (0.05 mg/kg) per 2-hr METH self-administration session for 11 consecutive days in Handled Control (n = 8) and Early Life Stress (n = 10). Data points represent group mean ± SEM. * $p \leq 0.05$ vs. Handled Control.

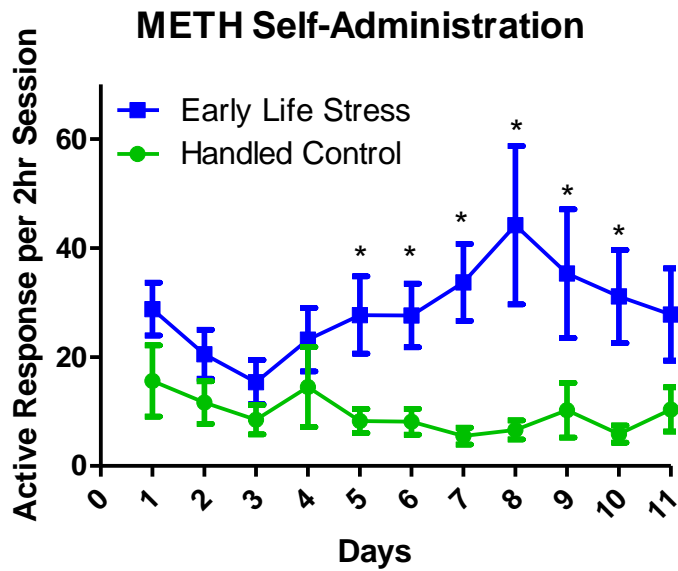


Figure 10. The Early Life Stress group emitted more active lever presses compared to the Handled Control group. Average number of active responses per 2-hr METH self-administration session for 11 consecutive days in Handled Control (n = 8) and Early Life Stress (n = 10). Data points represent group mean ± SEM. * $p \leq 0.05$ vs. Handled Control.

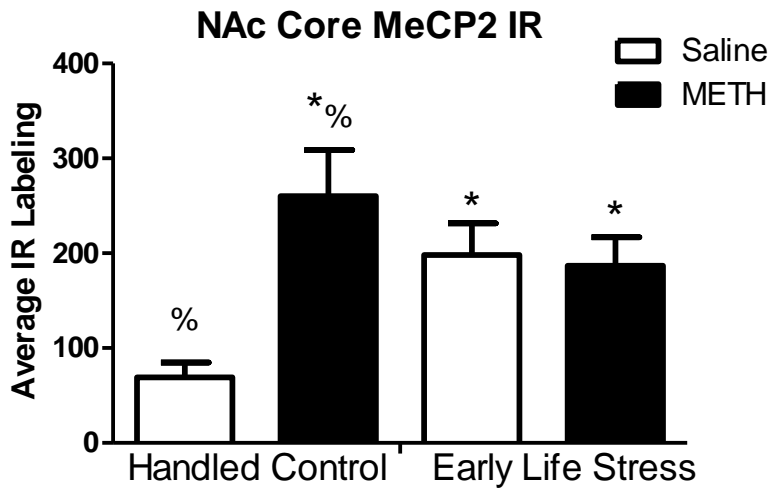


Figure 11. Cell counts for MeCP2 immunoreactivity in the NAc core per .63 mm² for Handled Control Saline (n = 5), Handled Control METH (n = 5), Early Life Stress Saline (n = 6), and Early Life Stress METH (n = 7) rats. Data points represent group mean ± SEM. **p* < 0.05 vs. Handled Control Saline and %*p* < .05 compared to ELS Saline.

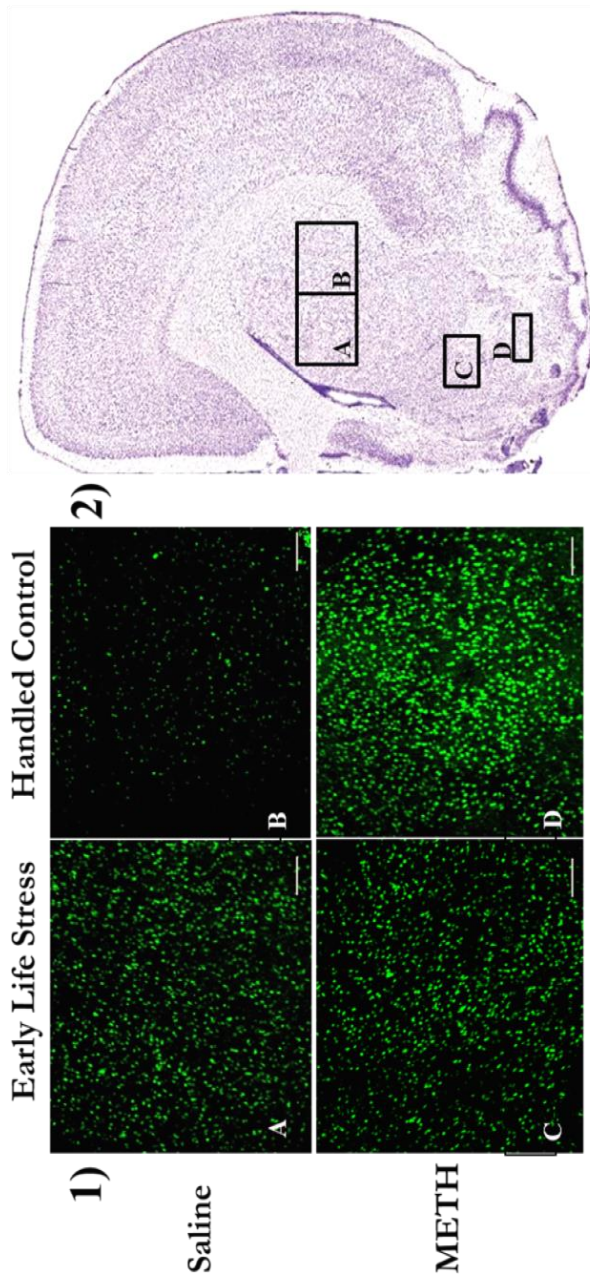


Figure 12. 1) Representative photomicrographs of MeCP2 immunoreactive labeling in the NAc core of A) Early Life Stress Saline, B) Handled Control Saline, C) Early Life Stress METH, and D) Handled Control METH in male rats. Scale bars 90 μ m. 2) Representative schematic of areas used for cell counts in A) Medial dorsal striatum, B) Lateral dorsal striatum, C) Nucleus accumbens core, and D) Nucleus accumbens shell.

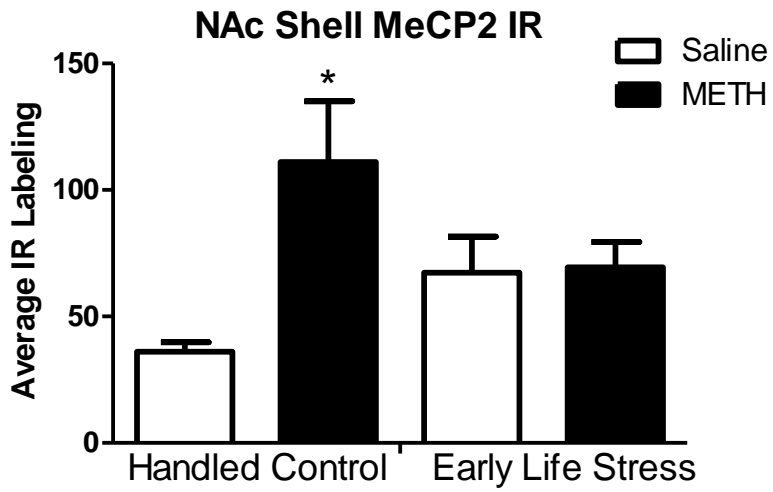


Figure 13. Cell counts for MeCP2 immunoreactivity per .28 mm² in the NAc shell for Handled Control Saline (n = 5), Handled Control METH (n = 5), Early Life Stress Saline (n = 6), and Early Life Stress METH (n = 7) rats. Data points represent group mean \pm SEM. * p < 0.05 vs. Handled Control Saline.

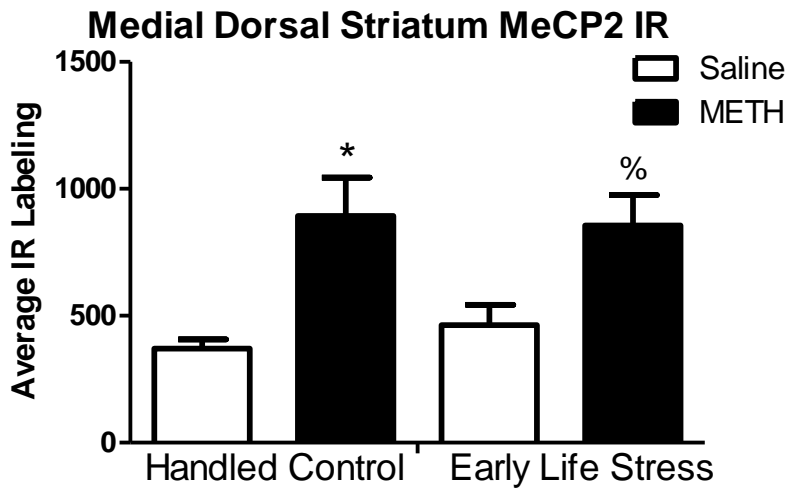


Figure 14. Cell counts for MeCP2 immunoreactivity per .22 mm² in the medial dorsal striatum for Handled Control Saline (n = 5), Handled Control METH (n = 5), Early Life Stress Saline (n = 6), and Early Life Stress METH (n = 7) rats. Data points represent group mean \pm SEM. * p < 0.05 vs. Handled Control Saline and % p < .05 vs. ELS Saline.

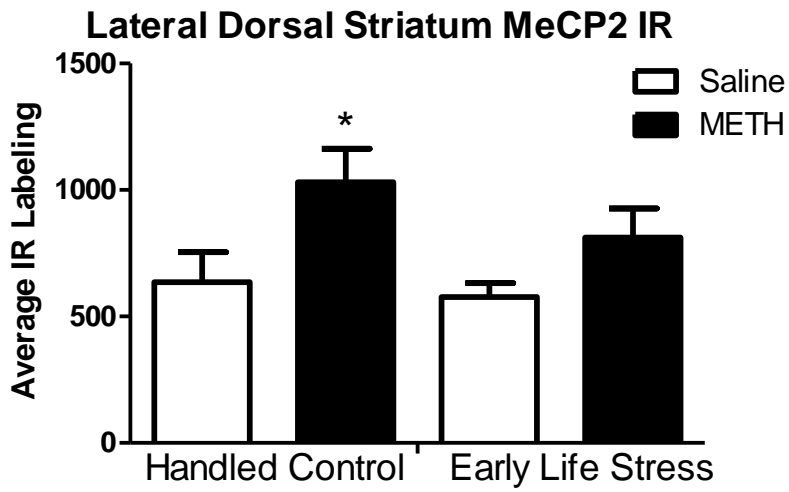


Figure 15. Cell counts for MeCP2 immunoreactivity per $.22 \text{ mm}^2$ in the lateral dorsal striatum for Handled Control Saline ($n = 5$), Handled Control METH ($n = 5$), Early Life Stress Saline ($n = 6$), and Early Life Stress METH ($n = 7$) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. Handled Control Saline.

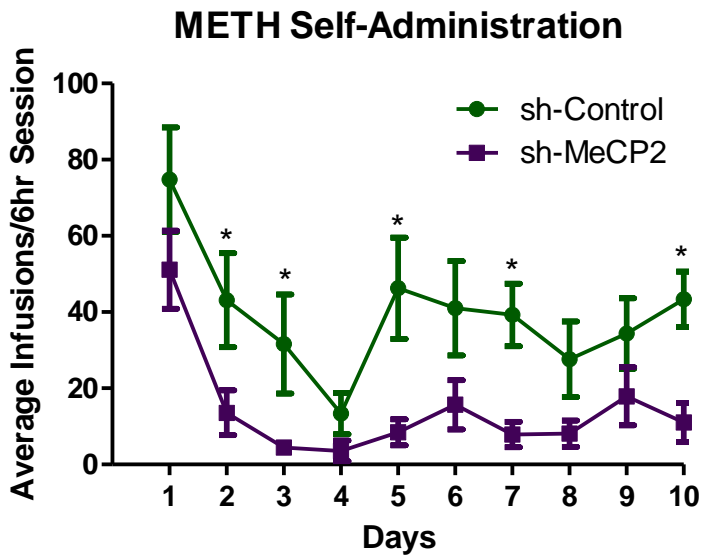


Figure 16. AAV-mediated knockdown of MeCP2 in the NAc core reduces METH intake in rats with extended access. Average number of infusions per 6-hr METH SA session for 10 consecutive days in sh-Control (n = 8) and sh-MeCP2 (n = 9). Data points represent group mean ± SEM. * $p \leq 0.05$ vs. sh-Control.

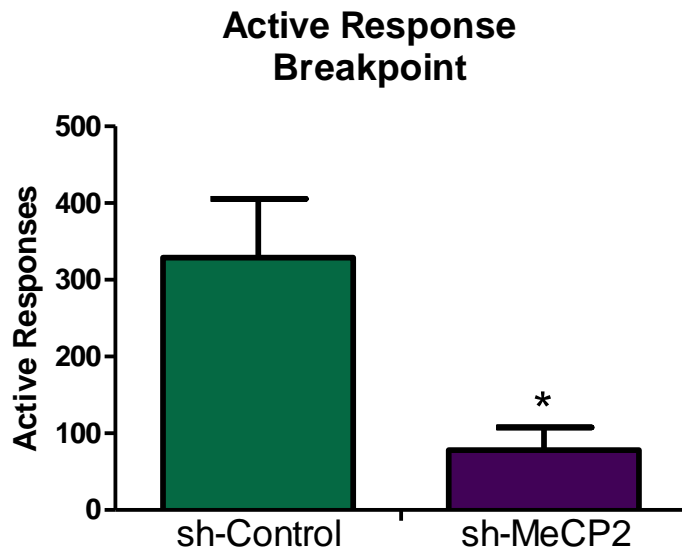


Figure 17. AAV-mediated knockdown of MeCP2 in the NAc core reduces lever pressing for METH under progressive ratio conditions. Sessions were terminated when no infusions were earned for 2 hrs. Average total number of active responses during progressive ratio tests following METH SA for sh-Control (n = 6) and sh-MeCP2 (n = 9). * $p < 0.05$ vs. sh-Control.

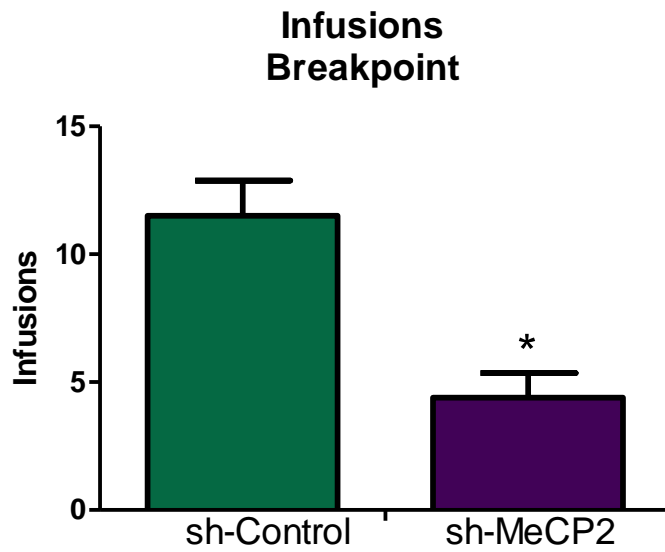


Figure 18. AAV-mediated knockdown of MeCP2 in the NAc core reduces the number of METH infusions earned under progressive ratio conditions. Sessions were terminated when no infusions were earned for 2 hrs. Average total number of infusions earned during progressive ratio tests following METH SA for sh-Control (n = 6) and sh-MeCP2 (n = 9). * $p < 0.05$ vs.sh-Control.

METH-Primed Reinstatement

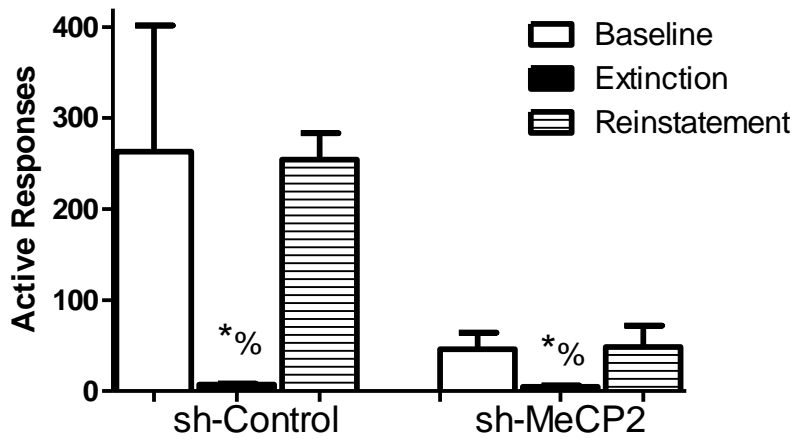


Figure 19. We observed no group differences in active lever responses during METH primed reinstatement. Rats received 1 mg/kg i.p. METH injection 15-min prior to placement in the operant chamber. Number of active responses across SA (FR1, e.g. Baseline, white bars), the final two days of Extinction training (black bars), and during drug-induced Reinstatement (striped bars) in sh-Control (n = 9) and sh-MeCP2 (n = 9) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. Baseline and % $p < 0.05$ vs. Reinstatement.

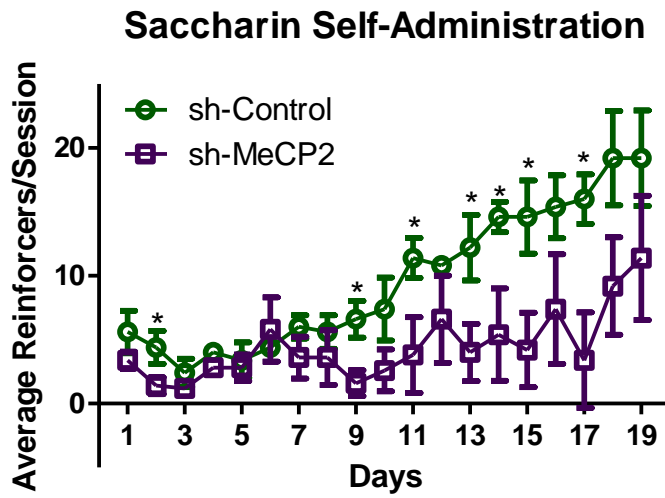


Figure 20. AAV-mediated knockdown of MeCP2 in the NAc core reduces saccharin intake. Average number of saccharin reinforcements earned per 30-min SA session for 19 consecutive days in sh-Control (n = 5) and sh-MeCP2 (n = 5). Data points represent group mean ± SEM. * $p \leq 0.05$ vs. sh-Control.

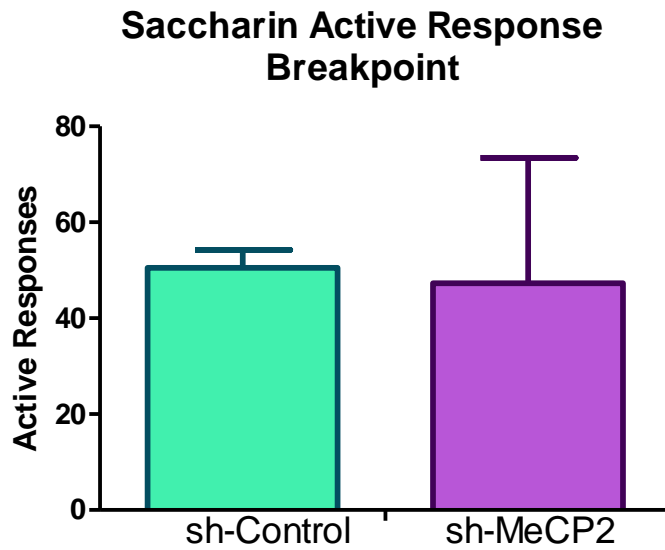


Figure 21. We observed no group differences in active lever responding for saccharin reinforcement under progressive ratio conditions. Average total number of active responses during progressive ratio tests following sacc SA for sh-Control (n = 5) and sh-MeCP2 (n = 5). No significant differences between sh-Control and sh-MeCP2 were observed.

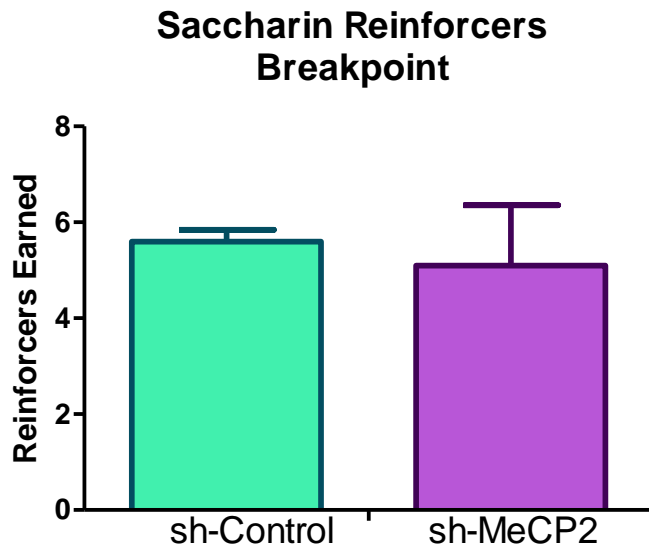


Figure 22. We observed no group differences in saccharin reinforcers earned under progressive ratio conditions. Average total number of reinforcers earned during progressive ratio tests following sacc SA for sh-Control (n = 5) and sh-MeCP2 (n = 5). No significant differences between sh-Control and sh-MeCP2 were observed.

Cue-Primed Reinstatement for Saccharin

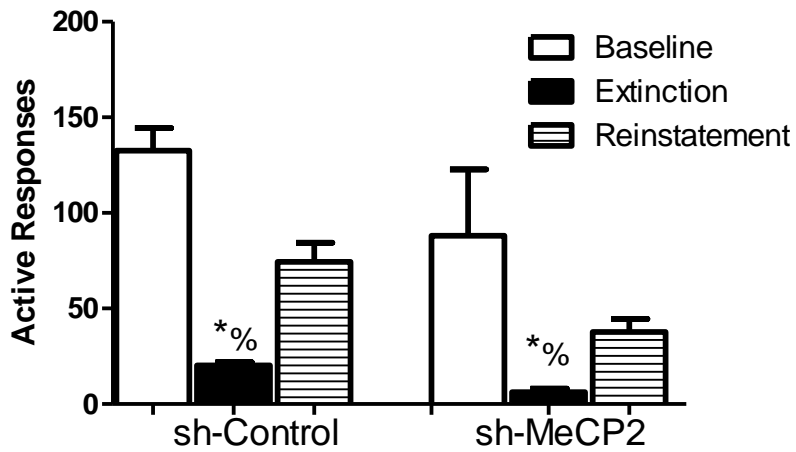


Figure 23. We observed no group differences in active lever responses during cue-primed reinstatement of saccharin seeking. Number of active responses across SA (FR1, e.g. Baseline, white bars), the final two days of Extinction training (black bars), and during cue-induced Reinstatement (striped bars) in sh-Control ($n = 5$) and sh-MeCP2 ($n = 5$) rats. Data points represent group mean \pm SEM. * $p < 0.05$ vs. Baseline and % $p < 0.05$ vs. Reinstatement.

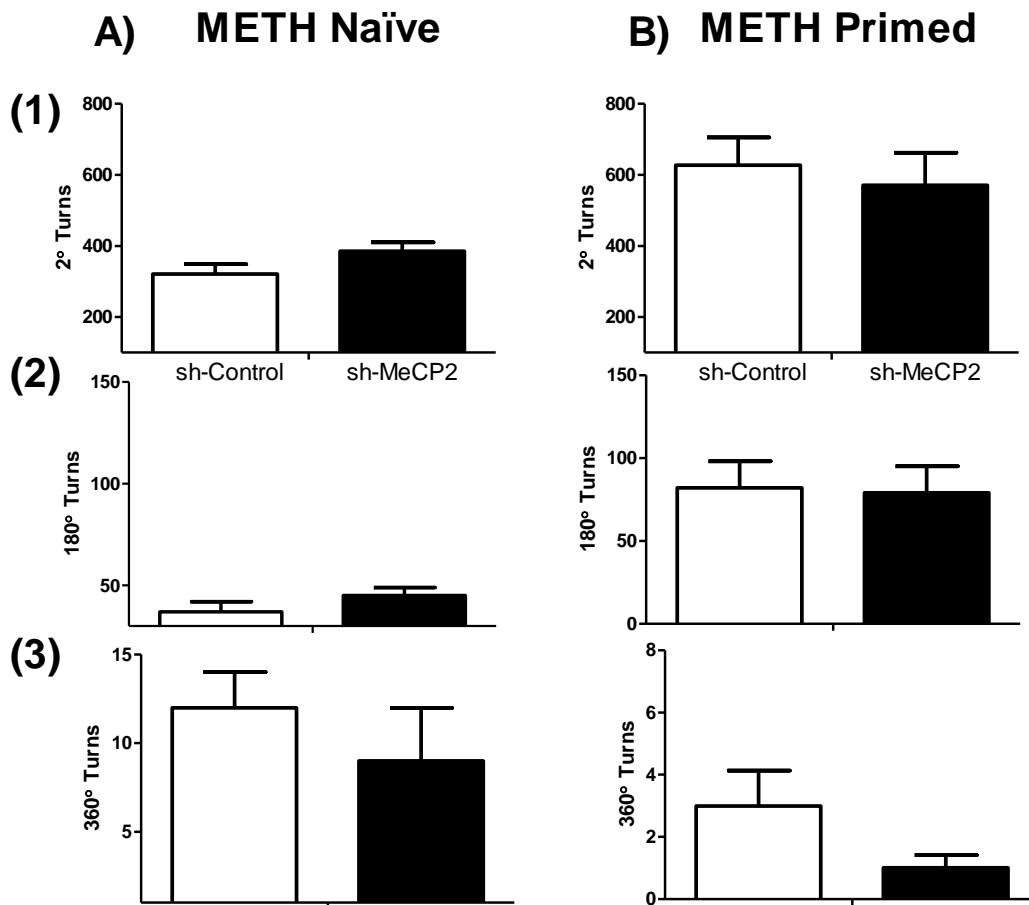


Figure 24. No significant differences between Control and MeCP2 knockdown groups were observed in rotational motor activity in A) METH naïve rats and B) METH primed rats (1mg/kg i.p injection 30-min prior to behavioral tests) 1 week post-reinstatement. Measured in (1) 2° turns, (2) 180° turns, and (3) 360° full rotations. Data points represent group mean \pm SEM.

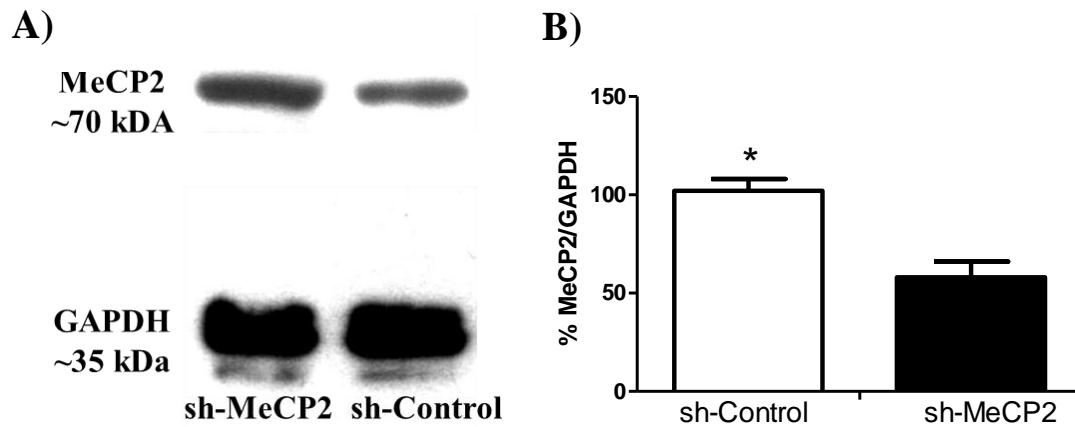


Figure 25. Virally-induced knockdown of MeCP2 expression in the NAc core. A) Representative bands of MeCP2 and GAPDH protein expression in AAV empty control virus (sh-Control) Vs. AAV expressing a short hairpin RNA against MeCP2 mRNA (sh-MeCP2), as determined by Western blot luminescence, in the NAc core. B) Mean % (\pm SEM) of MeCP2 luminescence in the NAc core in sh-Control (n = 4) and sh-MeCP2 (n = 4) * $p < .01$. Total knockdown was approximately 40%.

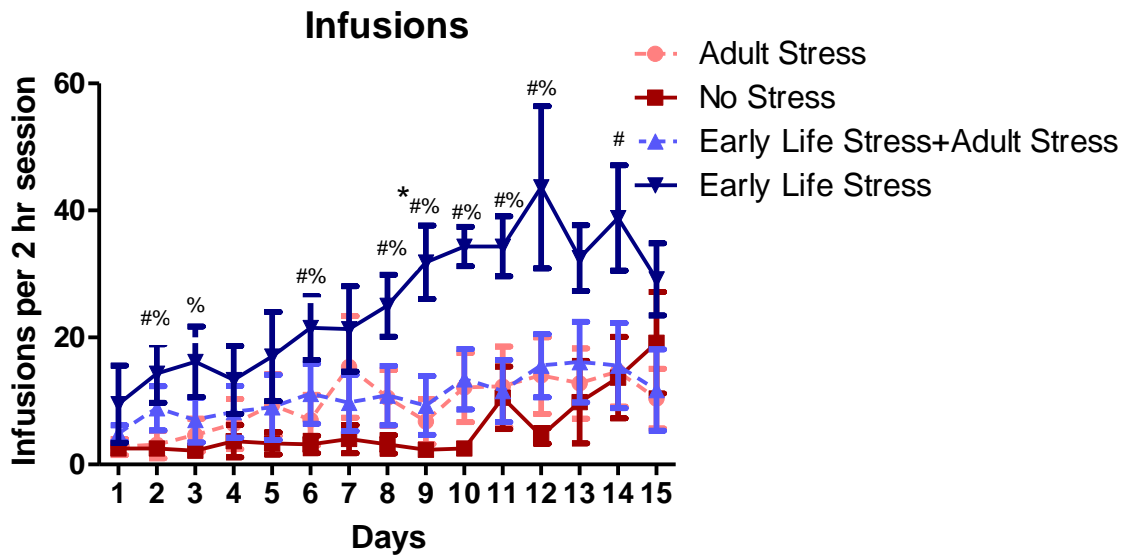


Figure 26. Early Life Stress animals self-administer more METH compared to other experimental groups. Average number of infusions per 2-hr METH SA session for 15 consecutive days in No Stress (n = 6), Adult Stress (n = 10), Early Life Stress (n = 6), and Early Life Stress + Adult Stress (n = 5) rats. Data points represent group mean \pm SEM. * $p \leq 0.05$ vs. Early Life Stress + Adult Stress, # $p \leq 0.05$ vs. Adult Stress, and % $p \leq 0.05$ vs. No Stress.

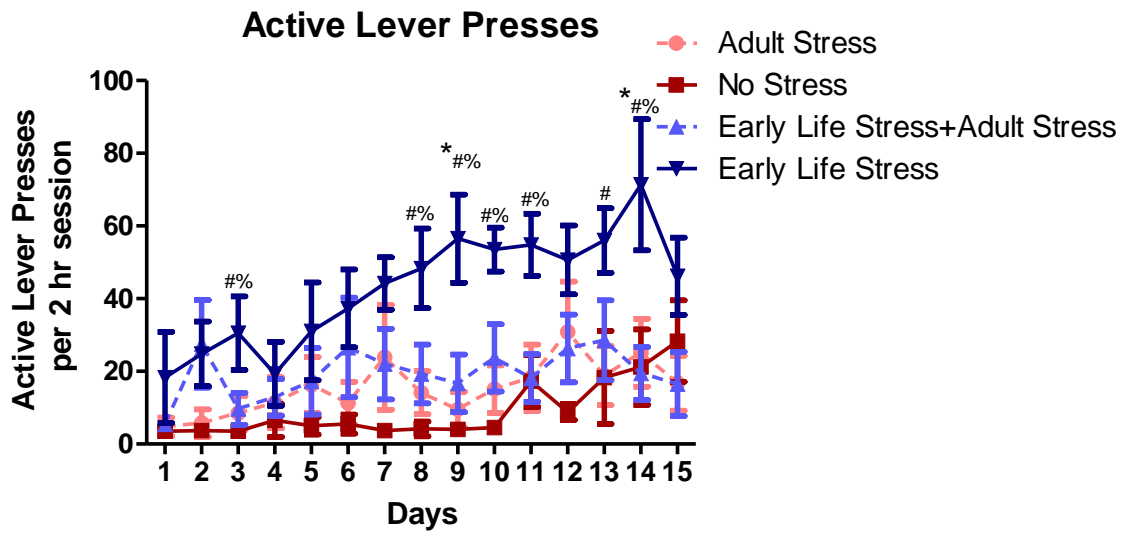


Figure 27. Early Life Stress animals exhibit more lever presses for METH reinforcement compared to other experimental groups. Average number of active responses per 2-hr METH self-administration session for 15 consecutive days in No Stress (n=6), Adult Stress (n=10), Early Life Stress (n=6), and Early Life Stress + Adult Stress (n=5) rats. Data points represent group mean \pm SEM. * $p \leq 0.05$ vs. Early Life Stress + Adult Stress, # $p \leq 0.05$ vs. Adult Stress, and % $p \leq 0.05$ vs No Stress.

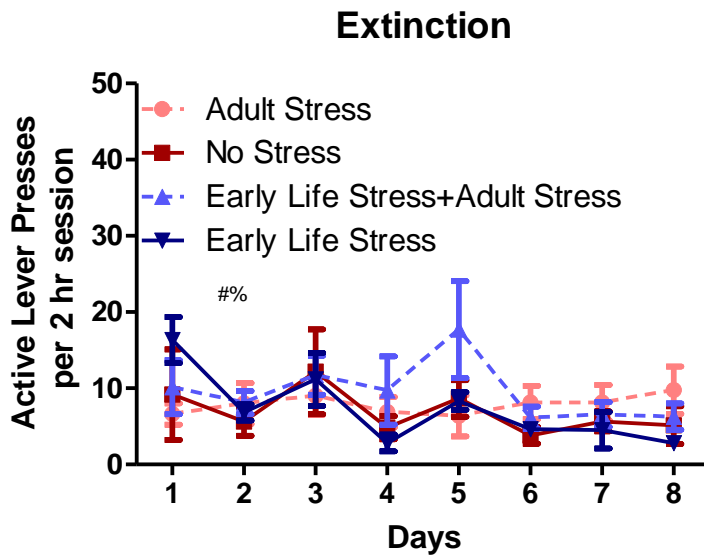


Figure 28. No significant group differences were observed in active lever presses during extinction training. Average number of active responses per 2-hr Extinction session for 8 consecutive days in No Stress (n = 6), Adult Stress (n = 10), Early Life Stress (n = 6), and Early Life Stress + Adult Stress (n = 5) rats. Data points represent group mean \pm SEM.

Cue-Induced Reinstatement

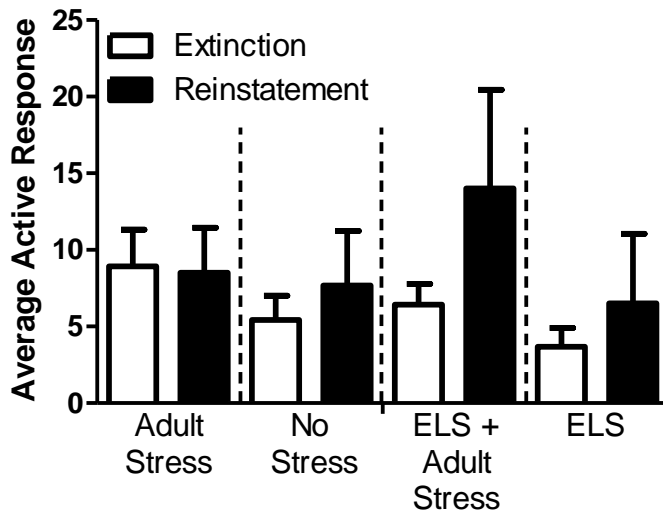


Figure 29. No significant group differences were observed in active lever pressing during cue-induced reinstatement testing. Dashed lines separate experimental groups. Number of active lever presses across the final two days of extinction training (white bars) and during cue-induced reinstatement (black bars) in No Stress ($n = 6$), Adult Stress ($n = 10$), Early Life Stress ($n = 6$), and Early Life Stress + Adult Stress ($n = 5$) rats. Data points represent group mean \pm SEM. No significant group differences were observed.

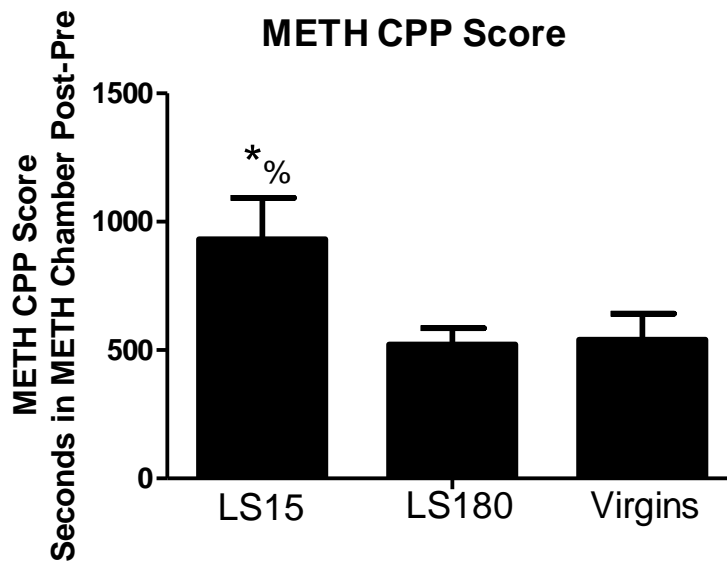


Figure 30. LS15 female rats displayed a significantly larger CPP magnitude compared to LS180 dams and Virgins. CPP score calculated by seconds spent in METH-paired chamber pre-conditioning subtracted from time spent in METH-paired chamber post-conditioning in LS15 (n = 8), LS180 (n = 10), and Virgins (n = 7). Data points represent group mean \pm SEM. * $p \leq 0.05$ vs. LS180 and % $p \leq 0.05$ Virgins.

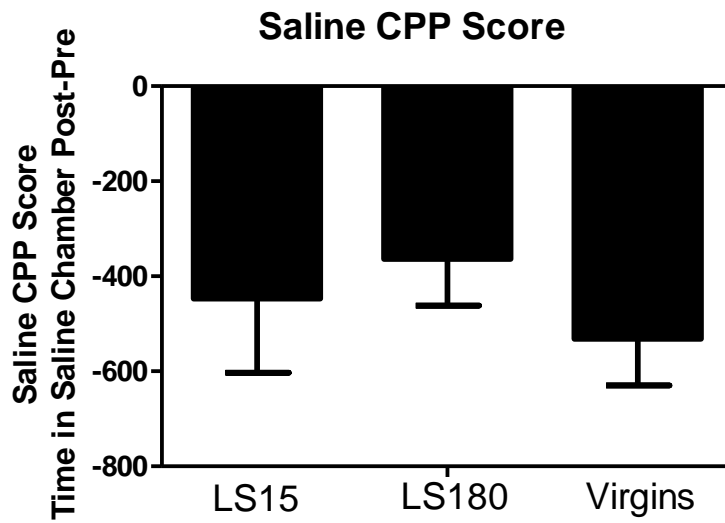


Figure 31. Saline CPP score calculated by seconds spent in Saline-paired chamber pre-conditioning subtracted from seconds spent in Saline-paired chamber post-conditioning in LS15 (n = 8), LS180 (n = 10), and Virgins (n = 7). Data points represent group mean \pm SEM. No significant differences between groups were observed.

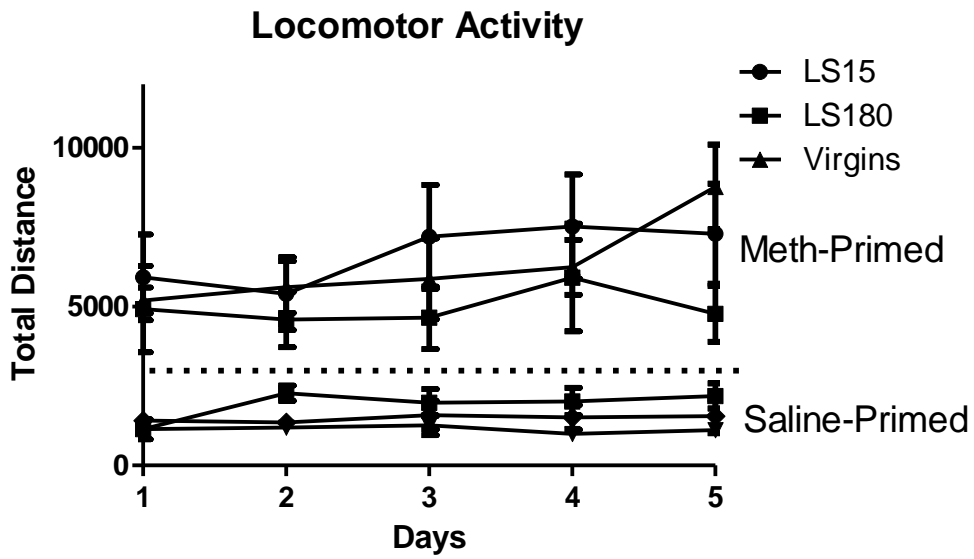


Figure 32. We observed no group differences in general locomotor activity across five conditioning days. Dashed line separates activity following METH or saline priming. Locomotor response to intraperitoneal Saline or METH (0.5 mg/kg) across five conditioning sessions in LS15, LS180, and Virgin female rats. No significant differences between groups were observed.