I give permission for public access to my thesis and for copying to be done at the discretion of the archive's library and/or the College library.

_____ _____

Signature Date

The Effect of Methadone Administration on Cocaine-Conditioned Hyperactivity and Sensitization in Mice

by

MaryElizabeth Simkevich

A Paper Presented to the

Faculty of Mount Holyoke College in

Partial Fulfillment of the Requirements for

the Degree of Bachelor of Arts with

Honor

Department in Neuroscience and Behavior

Mount Holyoke College

South Hadley, MA 01075

May 2019

****

To my parents John and Kathryn, and my sister Sarah

****

****

This paper was prepared

under the direction of

Professor André White

for eight credits

****

Acknowledgements

To *André White, PhD*., Thank you for giving me the opportunity to pursue research in your lab and for mentoring me over the past two years and helping me grow as a scientific researcher. When I first joined the lab, I was very unsure and shy about doing benchwork and never imagined that I would be confidently designing, executing, and troubleshooting my own experiments, or to top it off, writing a thesis. You have constantly supported and encouraged me in my endeavors, and I cannot thank you enough.

To *Jared Schwartzer PhD*., Thank you for serving on my thesis committee and for your unrelenting support and encouragement. Although we only met this spring, I am so glad that you encouraged me to take your psychopharmacology class, because it is one of my favorite classes I've taken. Whether it was helping me perform and interpret statistics or providing insight and feedback on my writing, you have gone above and beyond to help me through this process, and for that I am immensely grateful.

To *Amy Hitchcock Camp, PhD*., Thank you for serving on my thesis committee and for supporting my work. You made cell and molecular biology class engaging and fun, and I enjoyed having you as my professor. Thank you for being one of my last advisors at Mount Holyoke and sharing in the culmination of my undergraduate experience.

To *Ken Colodner*, PhD., Thank you for introducing me to neuroscience and for being one of the best advisors and professors I have had at Mount Holyoke.

To *Katie Byrne*, Thank you for always being a helpful resource and someone that I could go to regarding lab things.

To *the White Lab*, *especially Shuyi Qi and Julia Hecking,* Thank you for your constant encouragement and support. Shuyi, thank you for always being willing to come in and assist me this semester, even if it meant spending late nights in lab. Julia, thank you for always being willing to listen, laugh, and share in the ups and downs of our research as co-inaugural members of the White lab.

To *Clara Wang*, Thank you for being the best roommate and thesis buddy I could ever ask for. I feel so incredibly proud and humbled by your achievements, and I am glad that we got to write our theses together. Most of all, thank you for your friendship these past four years.

To *Gabi Muir, Evvie Halpert, Katie Dick, and Toni Soto,* Thank you for being my best friends at Mount Holyoke. Your encouragement and kindness have carried me through college.

To *Mom, Dad, and Sarah,* Thank you for your unconditional love and support throughout this very challenging year. Thank you for checking and listening to me talk about my research, even if you didn't always understand. I could not have done this without you.

To the *Mount Holyoke College Department of Biological Science and Neuroscience and Behavior Program,* Thank you for providing the resources, funding, and opportunities for me to pursue this independent research.

To *Dr. Marilyn Sarles and the Ellen P. Reese Fund*; thank you for your financial support through the LYNK-UAF program. Your contributions helped to make this project a reality.

To *the mice*, thank you for sacrificing your lives. I promise that I will never take them for granted.

Table of Contents

List of Figures viii
List of Tables x
Abstract xi
Introduction 12
Substance Abuse: Impact and Prevalence 12
Background 14
Polydrug Use 14
Medication-Assisted Treatment 15
Limitation of Current Treatment Protocols 17
Effects of Psychostimulants and Opioids on the Reward Pathway 18
Interaction Between Cocaine and the Endogenous Opioid System 21
Models of Cocaine-Associated Behaviors: Sensitization and Conditioned Hyperactivity 22
Research Objectives 26
Materials and Methods 27
Subjects 27
Drugs 28
Apparatus 29
Group Assignment 30
Chronic Methadone Administration (Experiment 1 only) 30
Handling and Habituation 31
Cocaine Conditioning 32
Acute Methadone Administration (Experiment 2 only) 33
Conditioned Hyperactivity and Reinstatement Tests 33
Data Analysis 35
Results 38
Experiment 1 38

Cocaine Conditioning 38
Conditioned Hyperactivity Test 39
Reinstatement Test 40
Experiment 2 44
Cocaine Conditioning 44
Conditioned Hyperactivity Test 45
Reinstatement Test 46
Discussion 50
Overview 50
Experiment 1: Chronic methadone 51
Experiment 2: Acute methadone 54
Limitations and Future Directions 56
Conclusion 61
Appendix 62
Literature Cited 66

List of Figures

Figure 1. Age-adjusted rate of opioid overdose deaths from 2011-2016 13

Figure 2. Age-adjusted rates for stimulant-related drug overdose deaths from 2011-2016 13

Figure 3. Pharmacotherapies approved for treatment of opioid dependence and addiction 17

Figure 4. Opioids and cocaine activate dopaminergic pathways thought to underlie addiction and relapse.....19

Figure 5. The μ-opioid receptor 21

Figure 6. Four open field chambers 29

Figure 7. Treatment group assignments for Experiment 1 and 2.....30

Figure 8. Schematic of methadone administration procedure 31

Figure 9. Schematic of cocaine conditioning procedure.....32

Figure 10. Schematic of conditioned hyperactivity test procedure.....34

Figure 11. Schematic of reinstatement test procedure 34

Figure 12. Experiment 1 Schematic: chronic methadone administration and conditioned hyperactivity and sensitization paradigm.....36

Figure 13. Experiment 2 Schematic: acute methadone administration and conditioned hyperactivity and sensitization paradigm.....37

Figure 14. Average distance traveled for paired and unpaired mice across treatment groups throughout conditioning.....41

Figure 15. Average distance traveled for paired and unpaired mice when challenged with saline 42

Figure 16. Average distance traveled for paired and unpaired mice when challenged with cocaine (10mg/kg) 43

Figure 17. Average distance traveled for paired and unpaired mice across treatment groups throughout conditioning.....47

Figure 18. Average distance traveled for paired and unpaired mice when challenged with saline 48

Figure 19. Average distance traveled for paired and unpaired mice when challenged with IP cocaine (10 mg/kg) 49

Figure 20. Cocaine conditioned place preference and extinction paradigm.....60

List of Tables

Table 1. Number of subjects and average weight at start of Experiment 1 and 2 27

Table 2. Methadone and cocaine dosages for Experiment 1 and 2.....28

Table 3. Conditioning treatments for Experiment 1 group assignments 62

Table 4. Experiment schedule to investigate the effects of chronic methadone on cocaine hyperactivity and sensitization.....63

Table 5. Conditioning treatments for Experiment 2 group assignments 64

Table 6. Experiment schedule to investigate the effects of acute methadone on cocaine hyperactivity and sensitization.....65

Abstract

Substance abuse disorder is characterized by compulsive drug-seeking behavior regardless of negative consequences. Methadone, a synthetic opioid, has been used as an effective treatment to aid rehabilitating patients resist opioid relapse. One factor complicating opioid recovery is the frequent and concurrent use of cocaine during medication-assisted treatment (MAT) programs. Both methadone and cocaine act on the reward pathway of the brain to produce distinct and complementary changes in drug-associated behaviors and memories. There are open questions about the effects of opioid treatment drugs on cocaine-associated behavior and memory. To that end, this study investigated the effect of chronic and acute methadone administration on cocaine-conditioned hyperactivity and sensitization in mice. In the first experiment, mice received daily methadone injections before undergoing cocaine-conditioning. After conditioning, mice were tested for locomotor sensitization (a cocaine-associated behavior), conditioned hyperactivity (cocaine-associated memory) and reinstatement (reflecting susceptibility for relapse). For the second experiment, mice received a single acute dose of methadone before undergoing cocaine conditioning and testing for locomotor sensitization, conditioned hyperactivity and reinstatement. Results indicated that chronic methadone does not affect the rate of cocaine sensitization, conditioned hyperactivity, or reinstatement. However, a trend suggests that chronic methadone may affect the magnitude of locomotor activating effect of cocaine. Similarly, acute methadone was found to have no effect on cocaine sensitization or conditioned hyperactivity, but an interaction revealed that acute methadone enhanced cocaine reinstatement. These results could have important implications for the treatment of patients who continue to use cocaine while participating in MAT programs.

Introduction

Substance Abuse: Impact and Prevalence

The abuse of substances such as tobacco, alcohol, and illicit drugs costs the U.S.A. approximately $740 billion in expenses related to crime, health care, and lost work productivity (NIDA, 2018). The National Survey on Drug Use and Health (NSDUH), which annually surveys civilian noninstitutionalized populations aged 12 and older, reports that in 2016 there were an estimated 28.6 million people aged 12 and older who currently used illicit drugs (SAMHSA, 2017). Of these, approximately 31 percent were under the age of 25 years old. Worryingly, drug overdose deaths have been increasing over the past decade, with these deaths frequently involving opioids such as heroin, oxycodone, morphine, and fentanyl, as well as stimulants such as cocaine and methamphetamine (Fig. 1 and 2, Hedegaard et al., 2018). In particular, the National Institute on Drug Abuse (NIDA) declared that the abuse of and addiction to opioids is a national public health crisis with over 130 people dying from opioid overdoses every day (2019). These increases in overdose-associated deaths highlight the ongoing need to better understand addiction and drug-abuse behavior in order to better address this public health concern.

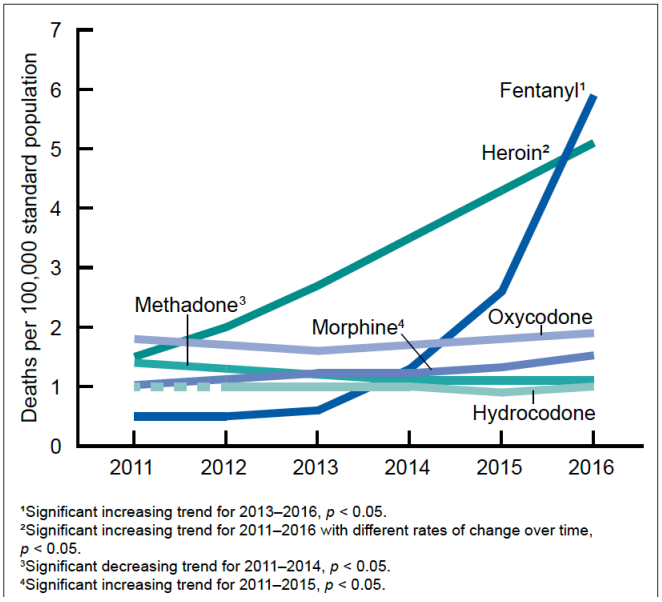

Figure 1. Age-adjusted rate of opioid overdose deaths from 2011-2016. The rate of overdose deaths involving heroin increased from 1.5 per 100,000 population to 5.1, climbing around 34% per year from 2011 through 2014, and approximately 20% per year from 2014 through 2016. This increase is second only to fentanyl-related overdose. Of these drugs, only methadone showed a decreasing overdose death rate (1.4 per 100,000 in 2011 to 1.1 per 100,000 in 2016). Adapted from Hedegaard et al., 2018.

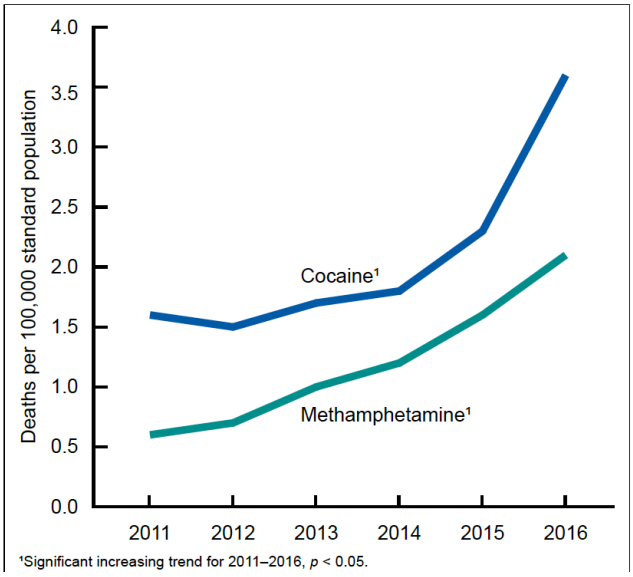

Figure 2. Age-adjusted rates for stimulant-related drug overdose deaths from 2011-2016. The rate of overdose deaths involving cocaine increased from 1.6 per 100,000 population to 3.6 from 2011 to 2016, with an 18% average increase per year. The rate of methamphetamine-related drug overdose deaths more than tripled from 0.6 per 100,000 population in 2011 to 2.1 in 2016 with an average yearly increase of around 29%. Adapted from Hedegaard et al. (2018).

Background

Substance abuse is defined as a chronic, relapsing behavioral disorder that is characterized by compulsive drug use and drug-seeking behavior despite negative consequences (Hyman, 2005). This disorder is partly caused by drug-associated changes to brain circuitry involved in learning, memory, reward, and motivation (Kreek et al., 2012). Risk factors for substance dependence and abuse include both genetic and environmental factors, such as lack of parental supervision, poor social skills, drug availability, low community socioeconomic status, and prior exposure to other drugs (NIDA, 2018). Individuals who are substance-dependent often experience debilitating drug cravings (the compulsion to administer the desired drug) that persist during periods of drug abstinence, thereby increasing their risk of relapse.

Polydrug Use

Individuals with a substance abuse disorder often use drugs from different drug classes in combination or separately during the course of drug abuse: This phenomenon is termed polydrug use. Polydrug use is associated with increased persistence of substance abuse disorders, an increased risk of overdose, and challenges to substance abuse treatment (Lorvick, Brown, Lambdin & Comfort, 2018). The ten most frequently mentioned drugs were often found in combination with each other, and drug combinations often commonly involved drugs of different classes. For example, approximately 70% of deaths involving opioids, fentanyl or heroin (the top two drugs in 2016) involved at least one other drug (Hedegaard et al., 2018).

Medication-Assisted Treatment

Due to the complexity of this disease, treatment often involves a combination of behavioral and pharmacological therapies such as medication. Medication-assisted treatment (MAT), also known as maintenance therapy, can reduce incidents of opioid abuse and extend drug abstinence (Bart, 2012). The aim of medicated-assisted treatment is to reduce the impact of procuring and using an illicit drug, such as heroin, by replacing it with a legal opiate (Anderson & Kearney, 2000). This prevents withdrawal symptoms, blocks the euphoric effects of the drug of abuse, minimizes drug cravings, and subsequently allows a person to reintegrate as a functional member into society.

The US Food and Drug Administration (FDA) has approved three drugs for medication-assisted treatment: naltrexone, buprenorphine, and methadone (NIDA, 2018). These drugs target the mu, delta, and kappa opioid receptors, which are the three primary receptors of the endogenous opioid system. Naltrexone is a mu opioid receptor antagonist that blocks receptors and inhibits opioid ingestion by competitive binding but will trigger withdrawal symptoms in opioid-dependent individuals. Therefore, it is used to help individuals who have stopped taking opioids to stay drug free (Fig. 3, NIDA, 2018). Buprenorphine is a partial mu opioid receptor agonist and kappa receptor antagonist which reaches a ceiling effect at a moderate dose and is associated with a lower risk for abuse, addiction, and side effects compared to full opioid receptor agonists (Fig. 3, Veilleaux et al., 2010). Despite some advantages, buprenorphine is not as effective at retaining patients in treatment programs compared to full mu opioid receptor agonists such as methadone (Mattick et al., 2014).

Methadone is a synthetic opioid that is commonly used in the detoxification and maintenance of patients addicted to opioids such as heroin or morphine. It is a full mu opioid receptor agonist which may mimic endogenous opioids, enkephalins, and endorphins and affect the release of other neurotransmitters such as acetylcholine, norepinephrine, and dopamine (Fig. 3, Anderson & Kearney, 2000). Methadone has a 4-6 hour duration of action and a long half-life of approximately 24 hours (Grissinger, 2011). Because this drug is an opioid, it may produce a rewarding effect and is susceptible to addiction, overdose, and adverse reactions such as respiratory depression. Therefore, methadone must be administered daily under controlled conditions (i.e. in clinics). Despite these drawbacks, it has been found that patients on methadone had 33% fewer opioid-positive drug tests and were over 4 times more likely to stay in treatment compared to controls, and methadone was significantly more effective than buprenorphine and non-pharmacological approaches at retaining patients in treatment and suppressing heroin use (Mattick et al., 2009; Mattick et al., 2014).

Cross-tolerance and cross-dependence among various opiates are motivations for the use of methadone in detoxification and maintenance since its long half-life delays onset of opiate withdrawal symptoms and diminishes the severity of the symptoms when compared to heroin, a shorter-acting opiate (Anderson & Kearney, 2000). It is able to diminish withdrawal symptoms, reduce illicit opiate use, and encourage retention in treatment in a dose-dependent manner. However, it is not simply the replacement of an illegal opiate for a legal one. Unlike drugs of abuse, a stabilization dose can be achieved (most patients stabilize between 60-120 mg per day) and there is rarely a need to increase the dose due to tolerance (Bart, 2012).

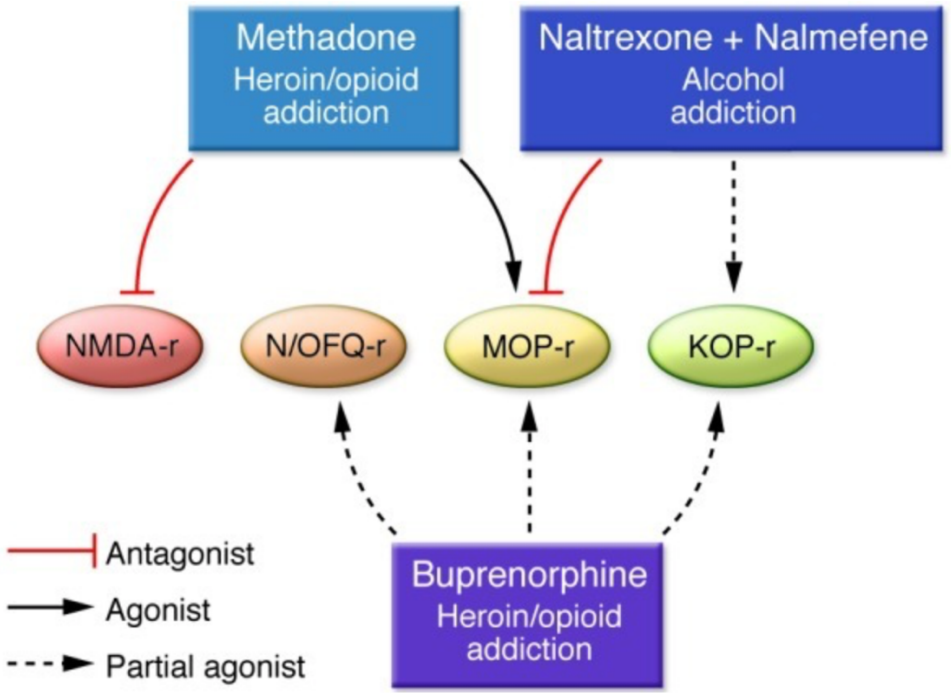

Figure 3. Pharmacotherapies approved for treatment of opioid dependence and addiction. Methadone is a full mu opioid receptor (MOP-r) agonist approved for chronic maintenance treatment of addiction to heroin or prescription opioids, as is the mu opioid receptor partial agonist buprenorphine. Naltrexone, also approved for the treatment of alcoholism and to prevent relapse to opioid dependence following detoxification, is a mu opioid receptor antagonist. Both buprenorphine and naltrexone also have affinity at the kappa opioid receptor (KOP-r), and buprenorphine is also a partial agonist at orphanin FQ/nociceptin receptors (N/OFQ-r), with relatively low potency. Adapted from Kreek et al (2012).

Limitations of Current Treatment Protocols

Treatment for polydrug use is further limited by current protocols. A common approach to classifying substance users at admission for treatment is to identify the primary drug of abuse. This allows for individualization of treatment, as many addictive drugs differ widely in their physiological and psychological effects. However, this approach fails to consider that patients may use more than one substance within the time period, which may complicate the overall substance abuse profile and lead to challenges in developing an effective treatment program (Brecht, Huang, Evans, & Hser, 2008). Several studies have reported that one third to more than

half of substance abusers (mainly heroin and cocaine users) have misused more than one class of addictive substances (Brecht et al., 2008). In fact, cocaine use by opioid addicts is a highly prevalent phenomenon and has acute medical and social effects (Leri, Tremblay, Sorge, & Stewart, 2004). For instance, cocaine administration has been frequently observed in both heroin addicts in methadone maintenance treatments before and during treatment (Leri, Bruneau, & Stewart, 2003). Researchers have reported cocaine use in 30-80% of heroin addicts not undergoing treatment and in 50-73% of heroin users undergoing methadone treatment (Leri, Bruneau, & Stewart, 2003). This high percentage of polydrug users, specifically for the combination of psychostimulant (cocaine) and central nervous system depressant (opioid) underscores a need to examine the pharmacological combination of these two drugs on addiction-related behaviors.

Effects of Psychostimulants and Opioids on the Reward Pathway

A shared feature of drugs of abuse is their ability to increase dopamine in the nucleus accumbens (Nac) (NIDA, 2018). The nucleus accumbens (NAc) is a region in the mesolimbic dopamine system involved in decision-making and motivation, especially in regard to pleasurable rewards. It is described as a limbic-motor interface which integrates the value of an expected reward with the motor action and behavioral response (Wenzel, Rauscher, Cheer, & Oleson, 2014; Morrison, McGinty, Hoffmann, & Nicola 2017). Dopamine release in the reward pathway is thought to affect the reinforcing effects of drugs of abuse, which leads to increased susceptibility to continued drug use and addiction. In particular, the dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is a major connection

that leads to increased reward (more precisely, "positive reinforcement") and addiction (Fields & Margolis, 2015).

Studies have also shown that both opioids and cocaine result in the activation of the dopaminergic mesocortical, mesolimbic, and nigrostriatal systems which induce regulatory changes (in mRNA or protein expression) that are thought to underlie chronic relapse in addictive disease (Kreek et al., 2012). However, psychostimulants and opioids modulate dopamine levels through different mechanisms. It is well-established that cocaine and other psychostimulants directly increase synaptic dopamine by inhibiting dopamine reuptake or increasing dopamine release from the cell, therefore prolonging the synaptic presence of dopamine and leading to increased activation (Fig. 4, Tilley et al., 2007; Leri, Bruneau, & Stewart, 2003).

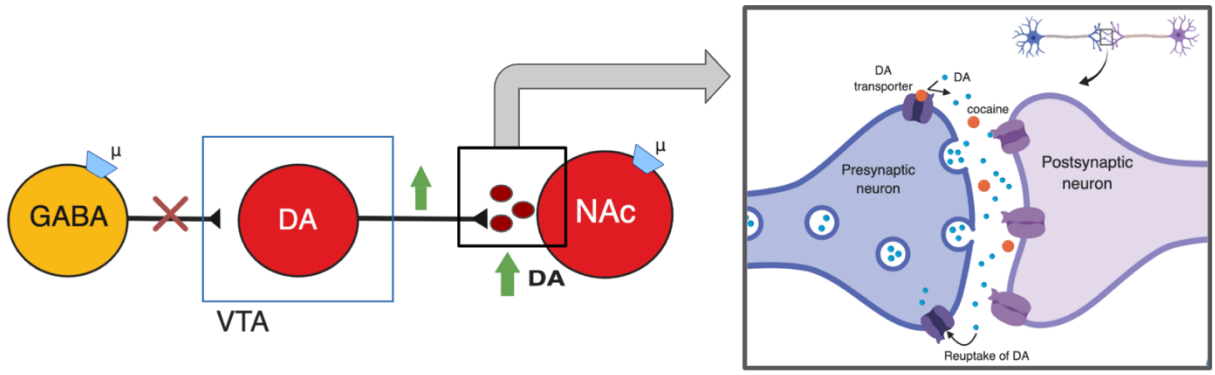

Figure 4. Opioids and cocaine activate dopaminergic pathways thought to underlie addiction and relapse. Activation of mu opioid receptors by opioids inhibits GABAergic interneurons which synapse with dopaminergic (DA) neurons in the ventral tegmental area (VTA), causing increased activation and dopamine release in the nucleus accumbens (NAc). Cocaine directly increases dopamine (DA) by binding to the dopamine transporter at the synapse and preventing reuptake, leading to increased levels of dopamine and signaling.

Opioids, meanwhile, act on the three major types of endogenous opioid receptors (mu, delta, and kappa) with varying effects. One particular study used in vivo microdialysis to examine the effects of the three opioid receptors on dopamine release in the NAc of rats (Spanagal, Herz, & Shippenberg, 1990). Researchers administered a mu opioid receptor agonist or a delta opioid receptor agonist and, using reverse high-performance liquid chromatography, found significantly increased dopamine levels (and its metabolites) in the nucleus accumbens, a region strongly implicated in decision making and cocaine addiction (Ostlund and Cui, 2018). Conversely, activation of the kappa opioid receptor had an inhibitory effect and lowered dopamine levels (Kreek et al., 2012). Additional research suggests that in substance abuse, mu receptor activation leads to the inhibition of GABAergic interneurons which normally inhibit dopaminergic neurons in the ventral tegmental area. This disinhibition of the dopaminergic VTA neurons results in an increased release of dopamine from VTA dopaminergic cells, which project to nucleus accumbens neurons (Fig. 4, Fields & Margolis, 2015). Furthermore, pharmacological and genetic manipulations have demonstrated that the mu opioid receptor, in particular, plays a role in mediating the therapeutic (analgesic) and aversive (addictive) effects of opioids (Fig. 5, Contet, Kieffer, & Befort, 2004). For instance, experiments using conditioned place preference and self-administration paradigms have shown that mu opioid receptor knockout mice do not become addicted to morphine or experience the analgesic effect of this drug (Contet, et al., 2004).

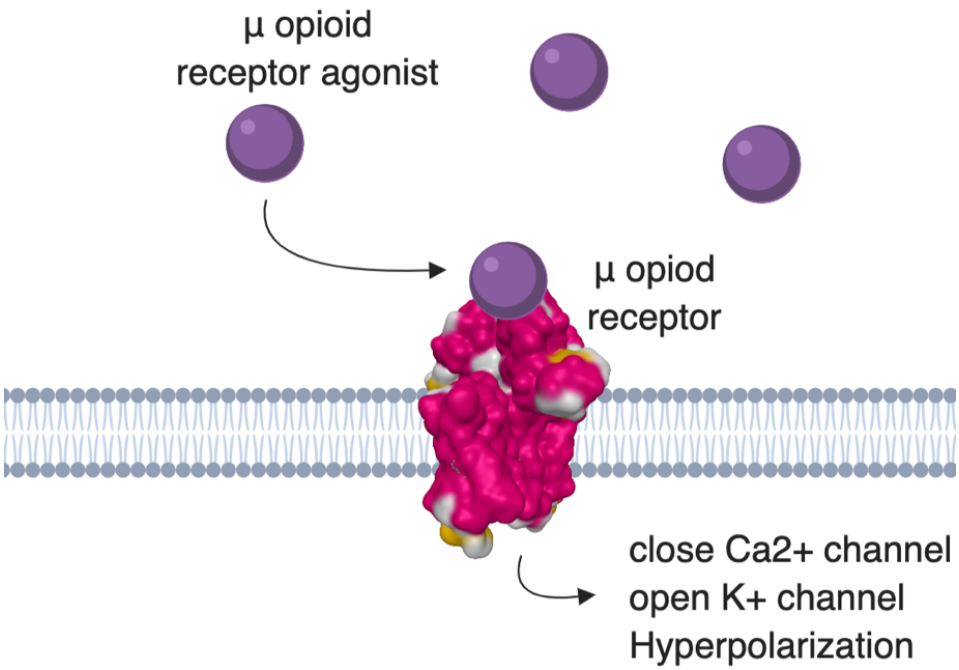

Figure 5. The μ-opioid receptor. Opioid agonists binding at mu receptors modulate intracellular effectors through inhibitory G proteins. Downstream effects are inhibitory, and include the closing of calcium channels, opening of potassium channels, and hyperpolarization of the neuron (Contet, et al., 2004).

Interaction between Cocaine and the Endogenous Opioid System

Not only is there co-localization between the endogenous opioid system and cocaine-associated dopaminergic pathways, but research suggests that these systems may regulate each other. For instance, intracerebroventricular microinjection of mu opioid receptor agonists have been shown to increase levels of dopamine and its metabolites in the nucleus accumbens (Ostlund & Cui, 2018; Spanagal & Shippenberg, 1990). However, direct injection of selective mu opioid receptor agonists, such as morphine, into the rat nucleus accumbens has been demonstrated to inhibit dopamine-induced hyperactivity, which suggests that the effects of mu

opioid receptor activation may either enhance or inhibit dopaminergic activity depending on the neural substrate (Erdo, Polgar, Mate, & Szekely, 1990).

In addition, cocaine administration appears to indirectly affect the endogenous opioid system. Chronic exposure to cocaine results in an upregulation of the kappa opioid receptor (KOR) and this KOR activation is thought to underlie aversion, or depression-like, anxiety-like states which are believed to negatively reinforce withdrawal from drugs of abuse and exacerbate relapse (Kreek et al. 2012). Furthermore, increases in mu opioid receptor levels in the nucleus accumbens and the dorsal striatum (caudate-putamen) after chronic cocaine exposure have been observed in rodent models of addiction (Burton et al., 2018), elucidating one potential molecular mechanism for the synergistic responses between opioids and cocaine.

This interaction between the mechanism of action of cocaine and the endogenous opioid system has implications for polydrug use treatment and suggests that opioid receptor activation might increase susceptibility to cocaine use. However, to date, there is little research examining the effects of long-term opioid receptor activation administration on cocaine-associated behaviors.

Models of Cocaine-Associated Behaviors: Sensitization and Conditioned Hyperactivity

Alterations in behavior after exposure to addictive drugs are a striking example of how chemical alterations to nervous system function produce long-lasting changes in behavior. Repeated drug administration can produce tolerance to the effects of some drugs but progressive enhancement with others. For instance, repeated intermittent administration of cocaine or other psychostimulants produces progressive increases in locomotor activity and stereotypic behaviors

in rodents and this phenomenon is referred to as behavioral sensitization or reverse tolerance (Smith, Penrod, Taniguchi, & Cowen, 2016; Chad et al. 2010). Much less is known about sensitization compared to tolerance, but it is theorized that the neuroadaptive changes governing the development of behavioral sensitization also underlie various facets of human drug dependence. It has been suggested that sensitization is due to non-associative changes in neural substrates that mediate uncontrolled drug effects and the development of behavioral plasticity and is associated with structural changes in brain regions such as the nucleus accumbens and prefrontal cortex (Robinson & Kolb, 2004; Zhang et al., 2015). In other words, behavioral sensitization is seen as a reflection of the increased pharmacological effect of the drug of abuse over time.

Sensitized locomotion in mice has been detected long after cessation of the drug of abuse (especially using psychostimulants) depending on the drug dose, location of administration, and length of exposure, and it provides an attractive model for studying the molecular and behavioral adaptations to addiction because it is a long-lasting process and because some forms of sensitization can be expressed in a context-dependent manner (Hyman, 2005). Studies have shown that the ventral tegmental area (VTA) is necessary for the initiation of behavioral sensitization (Kaur, 2004; Steketee & Kalivas, 2011). Repeated intracranial injections of amphetamine, a psychostimulant, into the VTA produce sensitization while injections into the nucleus accumbens produce locomotion (due to the direct effects of increased dopamine) but not sensitization. Additionally, amphetamine infusions into the VTA have also been shown to sensitize animals to peripheral administration of both amphetamine and cocaine, which highlights the importance of cross-tolerance in sensitization assays (Kaur, 2004).

Environmental stimuli, or "drug cues," associated with the drug of abuse can elicit physiological or psychological conditioned responses in addicts, even in the absence of the actual drug, and these Pavlovian responses are thought to contribute to continued drug-seeking behavior and relapse (Franklin & Druhan, 2000). According to classical conditioning theories of addiction, a stimulus may take on a new meaning by being associated with another stimulus (Otto, Ocleirigh, & Pollack, 2007). Conditioned hyperactivity is an associative learning process which can develop from repeated exposure to a psychostimulant, such as cocaine or amphetamine, and reflects the drug-associated memory and conditioned response. Rodents which have repeatedly received cocaine (the unconditioned stimulus) in a particular context (conditioned stimulus) will remember that context and form a conditioned response to that context (CS) when re-exposed to it. This is expressed by increased locomotor activity (conditioned hyperactivity) in these "Paired" animals, compared to animals which have not received the drug in that context ("Unpaired animals").

Studies have shown that the nucleus accumbens (NAc) is necessary for the expression of conditioned hyperactivity and other conditioned responses to stimuli associated with drugs of abuse. For example, one study showed that conditioned hyperactivity could be pharmacologically disrupted by infusing a GABA-B receptor agonist, baclofen, into the nucleus accumbens, or blocked by infusions of the GABA-A receptor agonist, muscimol, into the medial prefrontal cortex (Franklin & Druhan, 2000).

Behavioral sensitization and conditioned hyperactivity allow for the assessment of changes that develop over repeated exposure and the basic mechanisms of long-term changes in behavior using a behavioral sensitization and conditioned hyperactivity paradigm (Smith et al.,

2016). In our behavioral paradigm, two groups of rodents (paired and unpaired) are repeatedly exposed to the drug of abuse in two different contexts. The paired rodents are regularly exposed to a drug (such as cocaine) in an open-field locomotor activity chamber, while unpaired rodents receive the drug in their home cages. Over the conditioning period, paired rodents are expected to show progressively increased locomotor activity compared to unpaired rodents, and this demonstrates sensitization (which reflects the enhanced pharmacological effects of the drug). After conditioning, two tests evaluate conditioned hyperactivity (which reflects the cocaine-associated memory and conditioned response) and reinstatement (which evaluates the potential for relapse). During the conditioned hyperactivity test, all rodents are challenged with saline in the locomotor activity chambers, and paired rodents would be expected to show increased locomotor activity (a conditioned hyperactive response) compared to unpaired rodents. In the reinstatement test, all animals are exposed to cocaine, and the paired rodents are expected to show an enhanced response (context-specific sensitization) due to increased susceptibility to changes from the context-specific conditioning (White & Rauhut, 2014). Taken together, the progressive increases in locomotor activity after repeated drug exposure, as well as the enhanced response in animals re-exposed to a drug-associated environment serve as effective behavioral proxies to measure the pharmacological effects of poly-drug combinations, specifically cocaine and methadone, on drug-induced sensitization and conditioned hyperactivity.

Research Objectives

Drawing on previous research which has provided evidence for the co-localization and regulation of dopaminergic systems by opioids and cocaine, reports of cocaine use among patients in methadone maintenance programs, and studies which suggest that the ventral tegmental area plays a vital role in behavioral sensitization and that the nucleus accumbens is essential for conditioned hyperactivity, the purpose of this study was to examine how different methadone regimens may affect cocaine-associated behavior and memory.

Experiment 1 Objective: Examine the effect of chronic methadone on cocaine-associated sensitization and conditioned hyperactivity. I hypothesize that methadone will enhance sensitization but not conditioned hyperactivity and predict that conditioned hyperactivity will either undergo no change or decrease.

Experiment 2 Objective: Examine the effect of acute methadone on cocaine sensitization and conditioned hyperactivity.

Materials and Methods

Subjects

Male C57BL/6 mice were obtained from Jaxson Laboratories and the Schwartzer lab at Mount Holyoke College and single-housed a week prior to handling with OBEC's™ Premium WP bedding, and food and water available *ad libitum*. Mice were maintained at room temperature with a 12-hour light/dark cycle and all behavioral procedures were performed during the light cycle. Mice were 8-10 weeks old and the average weight at the start of the experiment was approximately 30.69 g for Experiment 1 and 30.23g for Experiment 2 (Table 1). All procedures were approved by the Mount Holyoke College Institutional Animal Care and Use Committee in accordance with the guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

	Number of Subjects (N)	Average Weight (g)
Experiment 1 (chronic methadone)	36	30.69 g
Experiment 2 (acute methadone)	18	30.23 g

Table 1. Number of subjects and average weight at start for Experiment 1 and 2.

Drugs

Drugs were obtained from Sigma Aldrich. Methadone was dissolved in saline and injected intraperitoneally (IP) at 0.20 mg/kg (body weight). This dose was derived from previous studies that examined the effect of chronic low-dose methadone on antidepressants and the rewarding effects of methadone using a mouse model (Schreiber et al., 2014; Holuj, Bisaga, & Popik, 2013). Similarly, cocaine was dissolved in saline and injected IP at 10 mg/kg. This dosage was derived from experiments by Smith et al. (2016) and Tilley et al. (2007) which examined cocaine-conditioned locomotor activity and sensitization in mice.

	Methadone (0.20 mg/kg)	Cocaine (10 mg/kg)
Experiment 1 (chronic methadone)	3 mg in 151 mL saline	66.53 mg in 66.53 mL saline
Experiment 2 (acute methadone)	0.06 mg in 3.0 mL saline	32.6 mg in 32.6 mL saline

Table 2. Methadone and cocaine dosages for Experiment 1 and 2.

Apparatus

The experiment apparatus consisted of four, wooden open-field chambers (30 cm x 23 cm x 29 cm interior dimensions). A layer of Envigo 7097 Teklad ¼" corncob bedding was placed at the bottom of each chamber, and this bedding was swirled to disperse the scent and feces were removed between each session. The chambers were illuminated by overhead lights, and all locomotor activity was recorded using EthoVisionXT14 tracking software (Fig. 6). Data were analyzed with Microsoft Excel, SPSS, and GraphPad Prism.

Figure 6. Four open field chambers.

Group Assignment

Prior to the experiment, mice were randomly assigned to a cocaine conditioning group (Paired and Unpaired) and methadone exposure group (chronic methadone or saline) resulting in four treatment conditions: Saline-Paired, Methadone-Paired, Saline-Unpaired, Methadone-Unpaired (Supplemental Table 3 and 5, Fig. 7).

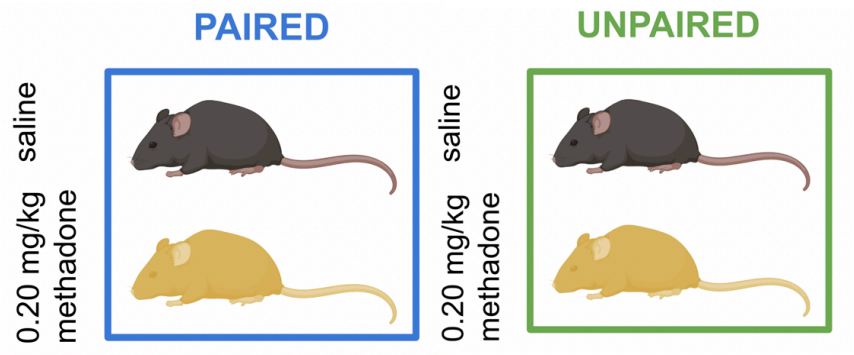

Figure 7. Treatment group assignments for Experiment 1 and 2. Experiment 1: Mice (n = 36) were assigned to saline-paired (n = 10) methadone-paired (n = 8), saline-unpaired (n = 10) or methadone-unpaired (n = 8) treatment groups. Experiment 2: As with the first experiment, mice (n = 18) were assigned to saline-paired (5), methadone-paired (n = 4), saline-unpaired (n = 5) or methadone-unpaired (n = 4) treatment groups.

Chronic Methadone Administration (Experiment 1 only)

Beginning 14 days prior to cocaine conditioning and continuing until testing (Day 1-22), both paired and unpaired mice received an intraperitoneal (IP) injection of methadone (0.20 mg/kg) or saline depending on group assignment. Methadone-paired and methadone-unpaired mice received methadone injections, while saline-paired and saline-unpaired mice received saline (Fig. 8, 12).

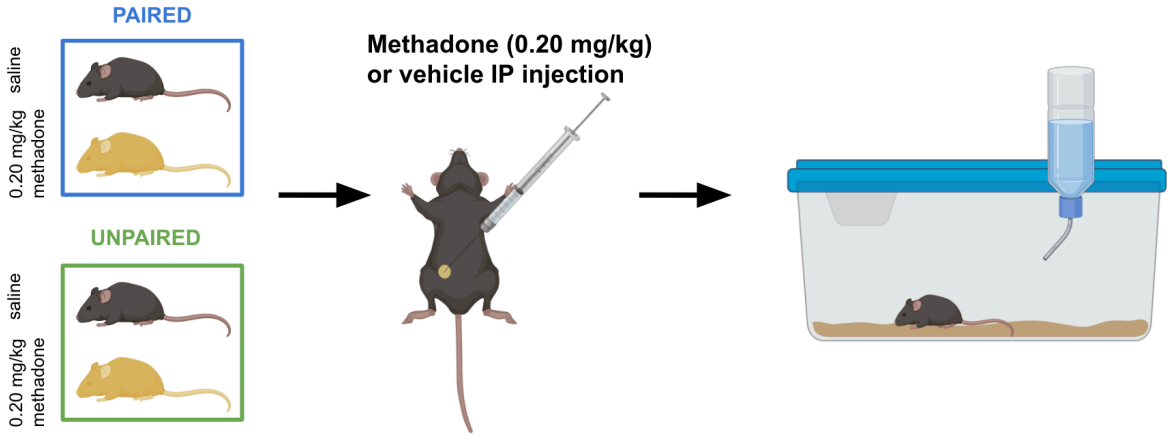

Figure 8. Schematic of methadone administration procedure. Mice were injected intraperitoneally (IP) with low dose methadone (0.20 mg/kg) or vehicle, depending on group assignment, and placed back into the home cage.

Handling and Habituation

To acclimate the mice to the locomotor chambers and reduce stress, mice underwent daily handling and habituation beginning three days prior to conditioning. Experiment 1: Six hours after the methadone (0.20 mg/kg) or saline injection each mouse was handled for two minutes (see Chronic Methadone, Fig. 12c). Experiment 2: mice were handled for two minutes each but did not receive IP methadone (0.20 mg/kg) or saline prior (Fig. 13c). Immediately after handling on the second and third day of this period, mice were placed in the locomotor chamber and allowed to freely explore while being video recorded for 30 minutes (Fig. 12c, 13b).

Cocaine Conditioning

Six hours after the IP methadone (0.20 mg/kg) or saline treatment (Fig.12d, 13d), paired mice were intraperitoneally injected with cocaine (10 mg/kg) while unpaired mice received an IP injection of saline and all animals were immediately placed in the locomotor chamber and allowed to explore freely while being video recorded for 30 minutes (Fig. 9a). The following day, paired mice were then exposed to saline while unpaired mice received an IP cocaine (10 mg/kg) injection and then all were placed back into their home cages (Fig. 9b). Mice continued to alternate daily between cocaine and saline exposures for a total of 8 days with each mouse always receiving cocaine in the same chamber type (home cage or locomotor arena; Fig. 9).

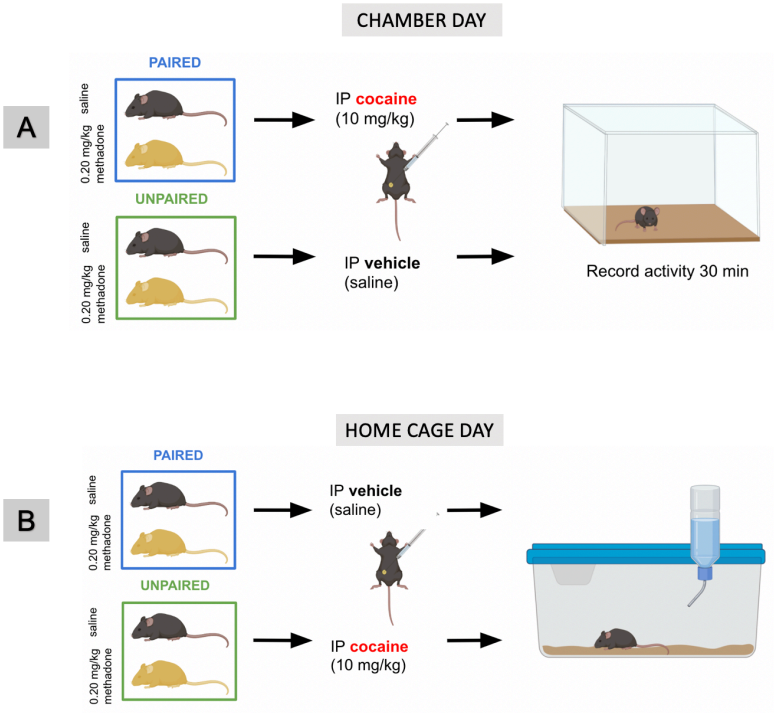

Figure 9. Schematic of cocaine conditioning procedure. Conditioning consisted of 4 alternating A) Chamber days and B) Home cage days for a total of eight days. A) On Chamber Days, paired mice received an IP cocaine injection (10 mg/kg) while unpaired mice received saline, then activity was recorded for 30 minutes in the locomotor chamber. B) During the alternating Home Cage days, paired mice received IP saline while unpaired mice received IP cocaine (10 mg/kg) injections and all mice were returned to their home cages.

Acute Methadone Injection (Experiment 2 only)

On the first day of cocaine conditioning (Day 4), mice received an acute IP injection of methadone (0.20 mg/kg) or saline. Methadone-paired and methadone-unpaired mice received methadone injections, while saline-paired and saline-unpaired mice received saline (Fig. 8). Six hours later, mice underwent conditioning, beginning with the Chamber Day procedure and alternating with the Home Cage day procedure (see Cocaine Conditioning, Fig. 9). The following conditioning days followed the same procedure as the first, but without the acute methadone injection (Fig. 13c).

Conditioned Hyperactivity and Reinstatement Tests

After the last day of conditioning, all mice were tested for cocaine-conditioned hyperactivity. During the Hyperactivity Test, all mice received an IP vehicle injection then placed in the locomotor box and recorded for 30 minutes (Fig. 10). The Reinstatement test was conducted the following day, and all mice received an IP cocaine (10 mg/kg) injection, were placed in the locomotor box, and recorded for 30 minutes (Fig .11). Sensitization was assessed by comparing Paired and Unpaired mice throughout conditioning. Cocaine-conditioned hyperactivity and reinstatement were evaluated by comparing Paired and Unpaired mice, which is an approach utilized by previous studies (White and Rauhut, 2014).

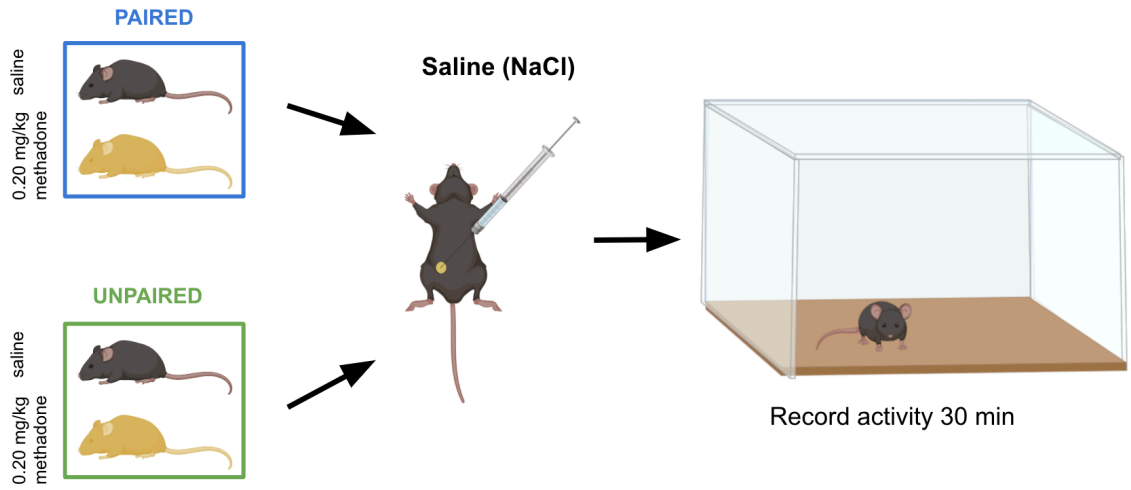

Figure 10. Schematic of conditioned hyperactivity test procedure. All mice were challenged with an IP injection of saline and locomotor activity was recorded in the open field chamber for 30 minutes.

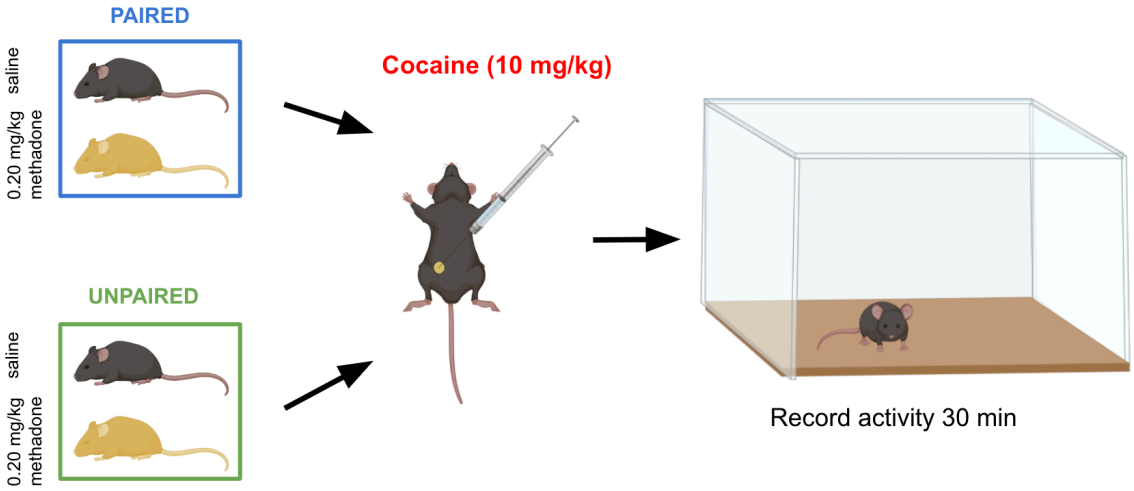

Figure 11. Schematic of reinstatement test procedure. All mice were challenged with an IP cocaine injection (10 mg/kg) and locomotor activity was recorded in the open field chamber for 30 minutes.

Data Analysis

Locomotor behavior was assessed based on total distance traveled using EthoVision XT 14. Data for each experiment were analyzed using Microsoft Excel, SPSS, G*Power, and GraphPad Prism. A three-way analysis of variance (ANOVA) was performed on the conditioning data to assess the effect of repeated measures effect Time and fixed effects Methadone and Cocaine on distance traveled across multiple conditioning days. Separate two-way ANOVAs with fixed effects were performed on Test Days 1 and 2 where the dependent variable was defined as Distance Traveled and fixed factors were defined as Methadone and Cocaine. Statistical decisions were made at α set to 0.05.

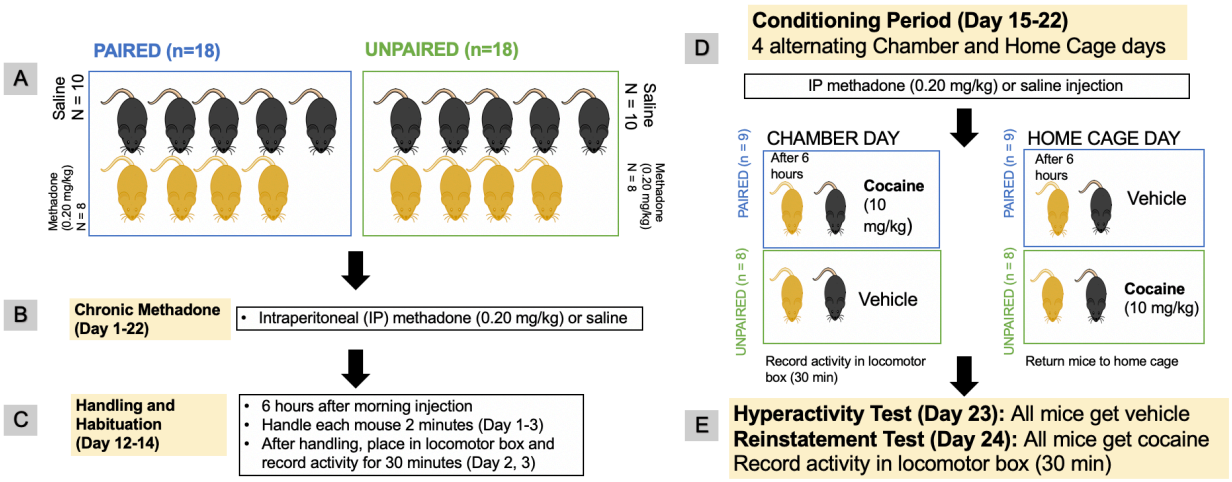

Figure 12. Experiment 1 Schematic: chronic methadone administration and conditioned hyperactivity and sensitization paradigm. (A) Mice were assigned to Paired and Unpaired groups with chronic methadone or vehicle in each group. (B) During a **Chronic Methadone period**, mice received an intraperitoneal (IP) methadone (0.2 mg/kg) or vehicle injection based on group assignment. (C) This was followed by the **Handling and Habituation Period:** 6 hours after methadone (0.20 mg/kg) or saline injection, mice were handled 2 min/day and then placed in the locomotor box for 30 minutes. (D) **Conditioning** consisted of 4 alternating Chamber and Home Cage days. On Chamber days, 6 hours after the methadone (0.20 mg/kg) or saline injection, Paired mice received IP cocaine (10 mg/kg) while Unpaired mice received saline, and all activity was recorded in a locomotor box for 30 minutes. On Home Cage days, 6 hours after IP methadone (0.20 mg/kg) or saline injection, Paired mice received IP vehicle, while Unpaired mice received cocaine (10 mg/kg). (E) In the **Conditioned Hyperactivity** test: all mice received IP vehicle, and activity was recorded for 30 minutes in the locomotor box. In the **Reinstatement** test: all mice received IP cocaine (10 mg/kg), and activity was recorded for 30 minutes in the locomotor box

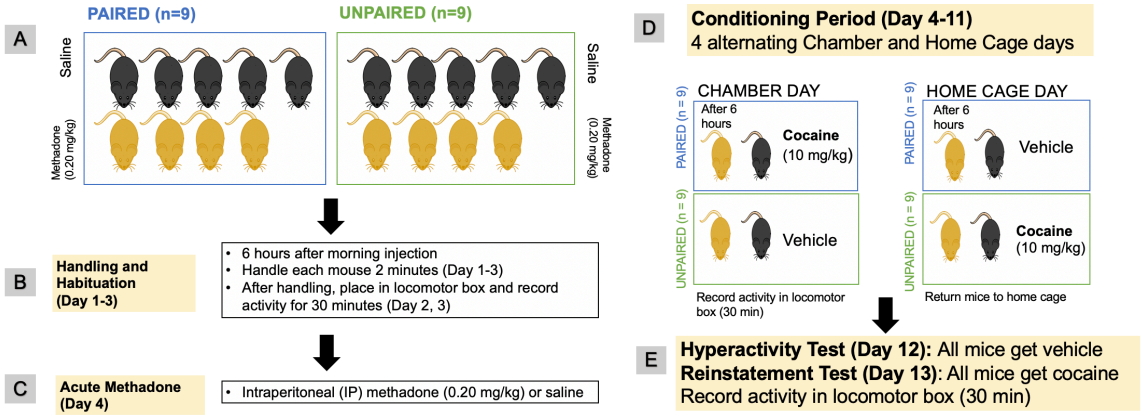

Figure 13. Experiment 2 Schematic: acute methadone administration and conditioned hyperactivity and sensitization paradigm. (A) Mice were assigned to Paired and Unpaired groups, with mice receiving chronic methadone and chronic vehicle in each group. (B) During a **Handling and Habituation Period:** all mice were handled for 2 min/day. (C) Mice received an intraperitoneal (IP) methadone (0.2 mg/kg) or vehicle (saline) injection based on group assignment on the first day of conditioning, 6 hours prior to cocaine administration. (D) **Conditioning** which consisted of 4 alternating Chamber and Home Cage days. On Chamber days, Paired mice received an IP injection of cocaine (10 mg/kg) while Unpaired mice received saline, and all activity was recorded in a locomotor box for 30 minutes. On Home Cage Days Paired mice received an IP injection of saline, while Unpaired mice received cocaine (10 mg/kg). (E) After conditioning, mice were tested for **Hyperactivity**: all mice received an IP vehicle injection, then placed in the locomotor box and recorded for 30 minutes. The experiment concluded with a **Reinstatement** test, where all mice received an IP cocaine (10 mg/kg), then placed in the locomotor box and recorded for 30 minutes.

Results

Experiment 1

Chronic methadone does not affect cocaine sensitization, conditioned hyperactivity, or reinstatement but trends suggest chronic methadone may enhance cocaine-associated locomotor activity in cocaine-paired mice, independent of time.

Cocaine Conditioning

Mice were assessed for total distance traveled during each conditioning day in the locomotor arena. A three-way analysis of variance (ANOVA) was to examine distance traveled with drug treatment (Methadone and Cocaine) as fixed effects and Time as a repeated measures effect (Fig. 14). The within-subjects factor, "Time," contained four levels corresponding to each chamber day (defined in SPSS as T1, T2, T3, T4) and between-subjects factors were "Methadone" and "Cocaine."

The within-subjects effects showed that there was a significant interaction between Time and Cocaine, with paired mice showing progressively increased locomotor activity compared to unpaired mice throughout the conditioning period $F(2.12, 65.7) = 10.574$, $MSE = 2156439$, $p = .000$, $\eta_p^2 = .25$. This enhanced locomotor activity in the paired mice (compared to unpaired mice) which increased over subsequent conditioning days is consistent with context-specific sensitization (Fig. 1, Chamber Day 1-4). In addition, a significant main effect for Time was observed $F(2.12, 65.7) = 7.41$, $MSE = 2156439$, $p = .001$, $\eta_p^2 = .19$, indicating that conditioning day had a significant effect on distance traveled. Specifically, the average distance traveled increased over subsequent conditioning days, but this effect is likely due to the increased activity observed in the paired mice. There were no interactions between Time and Methadone, $F(2.12,$

65.7) = 0.630, *MSE* = 2156439, $p = .545$, $\eta_p^2 = 0.02$ or between Time, Methadone, and Cocaine $F(2.12, 65.7) = 0.062$, *MSE* = 2156439, $p = .947$, $\eta_p^2 = 0.002$. This suggests that chronic methadone does not affect cocaine-associated sensitization (Fig. 14).

The between subjects effects indicated a moderate trend for an interaction between Methadone and Cocaine administration $F(1,31) = 2.11$, *MSE* = 7015711, $p = .157$, $\eta_p^2 = .06$ which suggests that, independent of time, chronic methadone may affect locomotor activity depending on cocaine treatment. A simple main effects analysis showed a trend for an effect of methadone ($p = .06$) as methadone-paired mice were more active than saline-paired mice on each day of conditioning, but unpaired mice did not differ in levels of locomotor activity regardless of whether they received methadone. There was also a significant main effect for Cocaine with paired mice showing increased locomotor activity compared to unpaired mice independent of time, $F(1,31) = 93.98$, *MSE* = 7015711, $p < .001$, $\eta_p^2 = .75$, and this reflects the direct, stimulating effects of cocaine. In addition, a significant main effect for Methadone was observed $F(1,31) = 4.91$, *MSE* = 7015711, $p = .034$, $\eta_p^2 = .137$, suggesting that chronic methadone enhances locomotor activity independent of time. However, since methadone did not affect distance traveled across both the paired and unpaired groups, but only in the paired animals, this suggests that these differences are being driven by the interaction (Fig. 14).

Conditioned Hyperactivity Test

After conditioning, all mice were challenged with saline to test for conditioned hyperactivity (Fig. 15). A two-way analysis of variance (ANOVA) with fixed effects was performed in SPSS where the dependent variable was defined as “Distance Traveled” and fixed factors were defined

as “Methadone” and “Cocaine.” There was no interaction between Methadone and Cocaine $F(1,31) = .00$, $MSE = 517971$, $p = .992$, $\eta_p^2 = .00$, which indicates that methadone does not affect cocaine-conditioned hyperactivity. However, the analysis revealed a significant main effect for Cocaine $F(1,31) = 12.67$, $MSE = 517971$, $p = .001$, $\eta_p^2 = .290$. Paired mice were significantly more active compared to unpaired mice, which reflects conditioned hyperactivity. In addition, there was a trend found for a main effect of Methadone $F(1,31) = 3.59$, $MSE = 517971$, $p = .068$, $\eta_p^2 = .10$. The trend showed that methadone-paired mice were slightly more active than saline-paired mice, which suggests that chronic methadone may increase locomotor activity independent of conditioning.

Reinstatement Test

Next, all mice were challenged with an IP injection of cocaine (10 mg/kg) to assess reinstatement (Fig. 16). As with the conditioned hyperactivity test, a two-way analysis of variance (ANOVA) with fixed effects was performed in SPSS where the dependent variable was defined as “Distance Traveled” and fixed factors were defined as “Methadone” and “Cocaine.” The analysis revealed a main effect of Cocaine $F(1, 31) = 8.90$, $MSE = 7707549$, $p = .006$, $\eta_p^2 = .22$, with cocaine-paired mice displaying significantly more locomotor activity compared to unpaired mice, demonstrating reinstatement and this suggests an increased susceptibility for relapse in the paired mice, which is consistent with current theories of sensitization. There was no main effect of Methadone $F(1, 31) = .59$, $MSE = 7707549$, $p = .446$, $\eta_p^2 = .02$, or interaction between Methadone and Cocaine $F(1, 31) = .01$, $MSE = 7707549$, $p = .934$, $\eta_p^2 = .00$, suggesting that chronic methadone does not affect reinstatement.

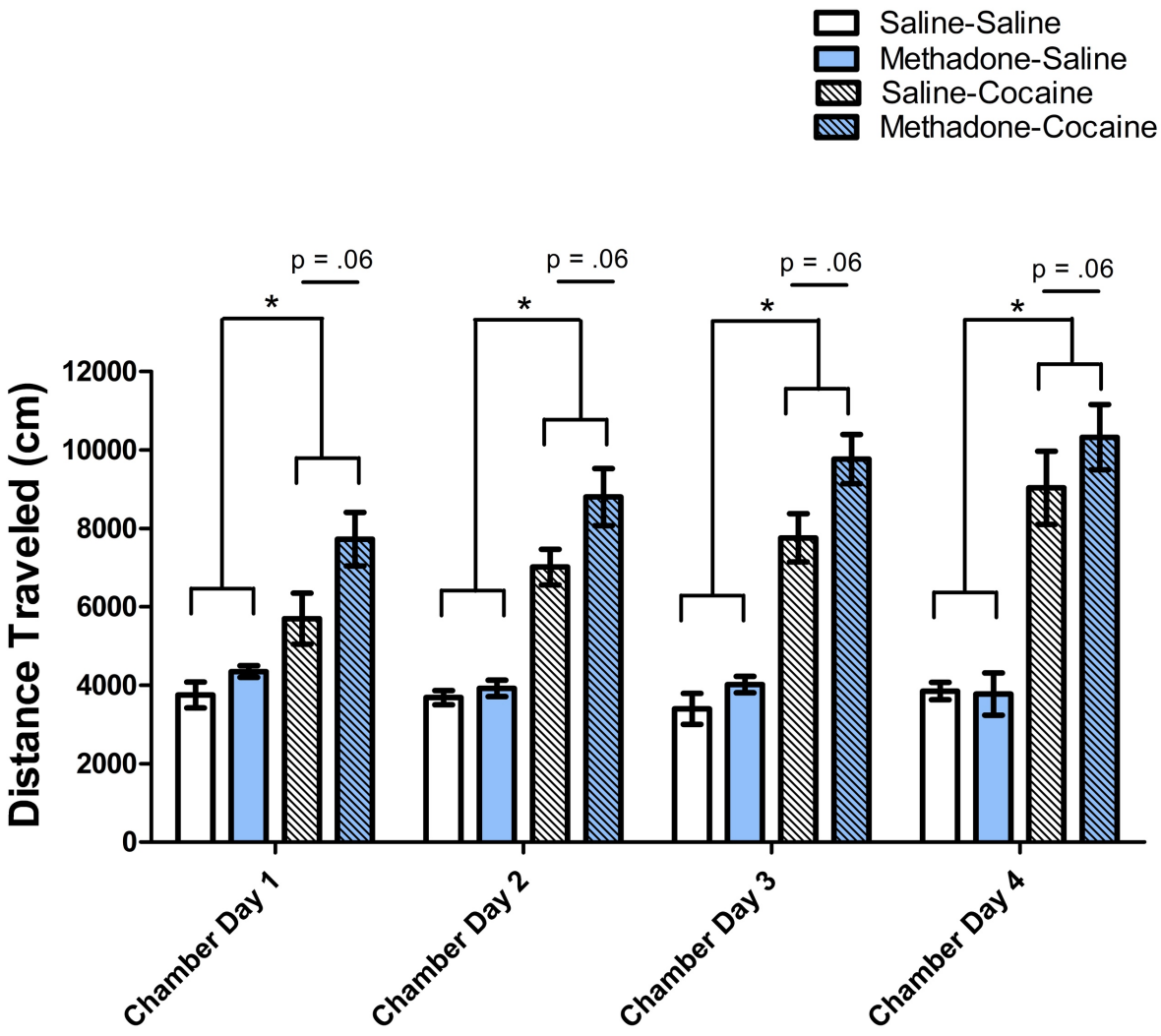

Figure 14. Average distance traveled for paired and unpaired mice across treatment groups throughout conditioning. * symbols denote significance ($p < 0.05$). There was a significant main effect of cocaine administration on distance traveled and paired mice were significantly more active than unpaired mice, and this increased over subsequent chamber days, reflecting sensitization ($p < .001$). There was a trend towards an interaction between methadone and cocaine independent of time, which suggests that chronic methadone may increase locomotor activity in cocaine-exposed mice, independent of cocaine-conditioning (p =.06).

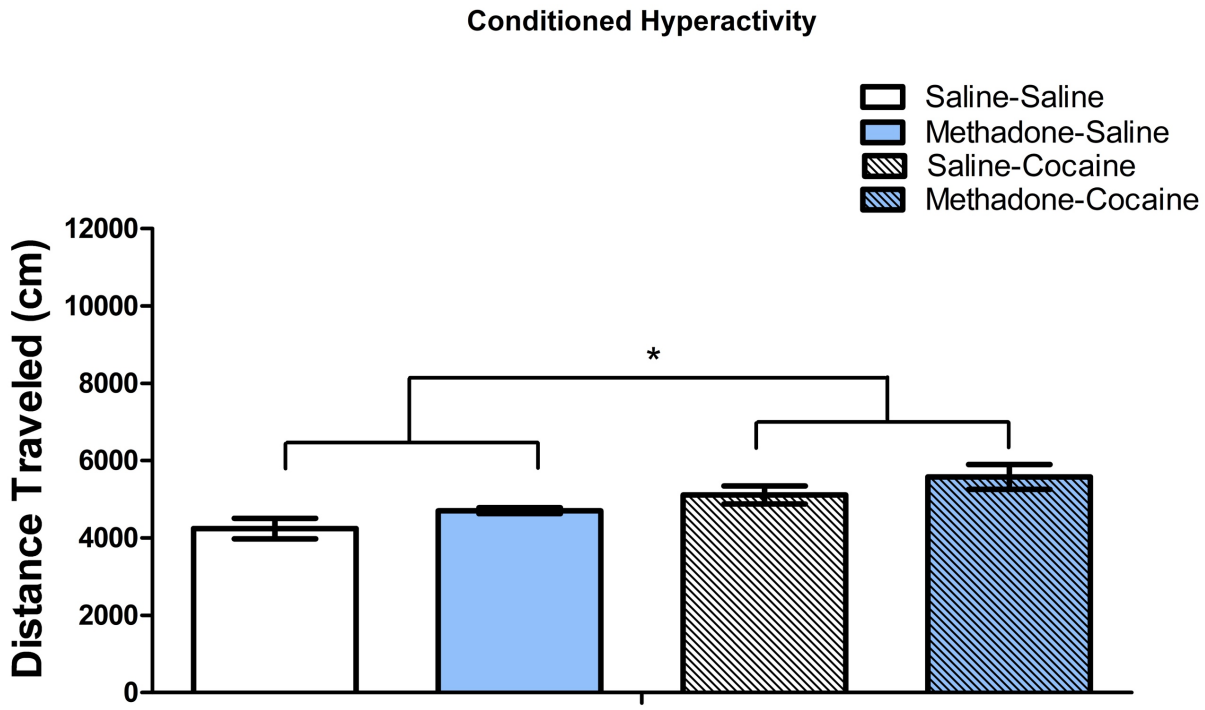

Figure 15. Average distance traveled for paired and unpaired mice when challenged with saline. * symbol denotes significance ($p < .05$). Paired mice were significantly more active compared to unpaired mice, which reflects conditioned hyperactivity ($p = .001$) but there was no interaction between methadone and cocaine ($p = .992$) suggesting that methadone does not affect cocaine-conditioned hyperactivity.

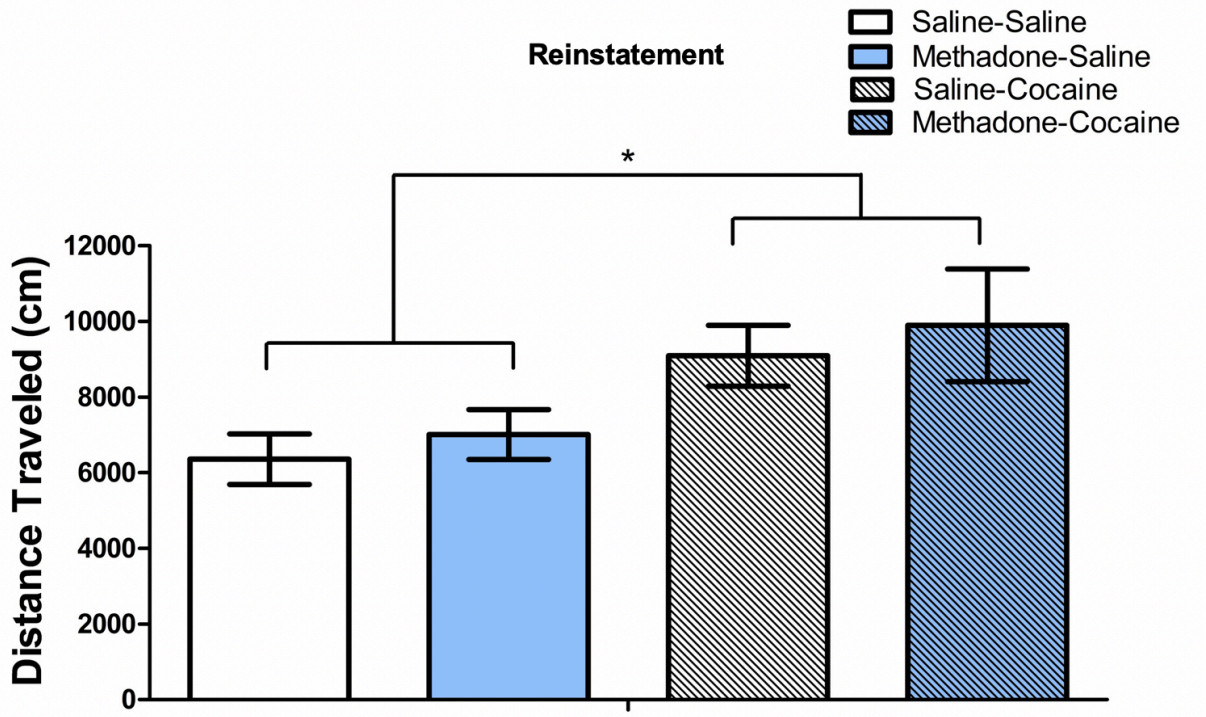

Figure 16. Average distance traveled for paired and unpaired mice when challenged with cocaine (10 mg/kg). * symbol denotes significance ($p < .05$). Paired mice were significantly more active than unpaired mice ($p = .006$), demonstrating reinstatement and suggesting an increased susceptibility to relapse. There was no main effect of ($p = .446$), or interaction between methadone and cocaine ($p = .934$), suggesting that chronic methadone does not affect reinstatement.

Experiment 2

Acute methadone administration does not significantly affect cocaine sensitization or conditioned hyperactivity but appears to enhance cocaine-induced reinstatement.

Cocaine Conditioning

In order to understand whether the results seen in Experiment 1 were due to the acute effects of methadone, or to the chronic administration regimen of the drug, I next assessed the effects of and acute dose of methadone on cocaine-associated sensitization and conditioned hyperactivity. Similar to the previous study, mice were assessed for distance traveled throughout conditioning period. A three-way analysis of variance (ANOVA) was to examine distance traveled with Methadone and Cocaine as fixed effects and Time as a repeated measures effect (Fig. 17). The within-subjects factor, "Time," contained four levels corresponding to each chamber day (defined in SPSS as T1, T2, T3, T4) and between-subjects factors were "Methadone" and "Cocaine."

Within-subject effects showed that there was a trend toward an interaction between Time and Cocaine $F(1.22, 14.61) = 3.95$, $MSE = 9106791$, $p = .06$, $\eta_p^2 = .25$. The trend indicates that paired mice displayed progressively increased locomotor activity compared to unpaired mice throughout the conditioning period and this enhanced locomotor activity in the paired mice is consistent with context-specific sensitization (Fig. 17, Chamber Day 1-4). There was a trend toward a main effect of Time on distance traveled $F(1.22, 14.61) = 3.34$, $MSE = 9106791$, $p = .082$, $\eta_p^2 = .22$, suggesting that chamber day may affect distance traveled. Overall, the average distance traveled increased over subsequent chamber days, but this effect was likely due to increased activity in the paired animals during conditioning. There were no interactions between

Time and Methadone, $F(2.12, 65.7) = 0.313$, $MSE = 2156439$, $p = .313$, $\eta_p^2 = 0.09$ or between Time, Methadone, and Cocaine $F(2.12, 65.7) = 0.591$, $MSE = 2156439$, $p = .486$, $\eta_p^2 = 0.05$. This suggests that acute methadone does not affect cocaine-associated sensitization (Fig. 17).

The between subjects effects showed a significant main effect of Cocaine, $F(1,12) = 12.09$, $MSE = 13269908$, $p = .005$, $\eta_p^2 = .50$. Independent of time, paired animals showed increased activity compared to unpaired animals and this reflects the stimulating effect of cocaine (Fig. 17, Chamber Days 1-4). There was no main effect of Methadone $F(1,12) = .11$, $MSE = 13269908$, $p = .742$, $\eta_p^2 = .01$, and no Methadone by Cocaine interaction $F(1,12) = .54$, $MSE = 13269908$, $p = .477$, $\eta_p^2 = .04$, suggesting that acute methadone does not affect cocaine-associated locomotion, independent of time.

Conditioned Hyperactivity Test

Next, all mice were challenged with saline to test for conditioned hyperactivity (Fig. 18). A two-way analysis of variance (ANOVA) with fixed effects was performed in SPSS where the dependent variable was defined as “Distance Traveled” and fixed factors were defined as “Methadone” and “Cocaine.” There was a trend toward a main effect for Cocaine $F(1, 12) = 3.43$, $MSE = 1026034$, $p = .089$, $\eta_p^2 = .22$, with paired mice displaying slightly more activity compared to unpaired mice, and this is consistent with cocaine-conditioned hyperactivity. There was no main effect of Methadone $F(1, 12) = .28$, $MSE = 1026034$, $p = .604$, $\eta_p^2 = .02$ and no Methadone by Cocaine interaction, $F(1, 12) = 1.11$, $MSE = 1026034$, $p = .313$, $\eta_p^2 = .09$, indicating that animals that received methadone were no different from controls in their

locomotor activity and suggesting that acute methadone administration does not affect cocaine-conditioned hyperactivity (Fig. 18).

Reinstatement Test

Finally, mice were challenged with cocaine (10 mg/kg) to assess locomotor responses to reinstatement (Fig. 19). A two-way analysis of variance (ANOVA) with fixed effects was performed using dependent variable "Distance Traveled" and fixed factors "Methadone" and "Cocaine." The analysis indicated that while there was no main effect of Methadone $F(1, 12) = 1.65$, $MSE = 7077369$, $p = .22$, $\eta_p^2 = .12$, there was a statistically significant interaction between Methadone and Cocaine $F(1, 12) = 5.05$, $MSE = 7077369$, $p = .044$, $\eta_p^2 = .30$. Specifically, Methadone-unpaired mice showed lower activity levels (M = 4891.76 cm) compared to saline-unpaired mice (M = 6215.65 cm), while methadone-paired mice showed higher activity (M = 10509.4 cm) compared to saline-paired mice (M = 5661.38 cm). In total, these data suggest that acute methadone exposure enhances cocaine-reinstated locomotion (Fig. 19). In addition, there was a trend toward a main effect of Cocaine $F(1, 12) = 3.39$, $MSE = 7077369$, $p = .090$, $\eta_p^2 = .22$ however, this effect is likely driven by the interaction between Methadone and Cocaine and the increased activity of the methadone-paired group.

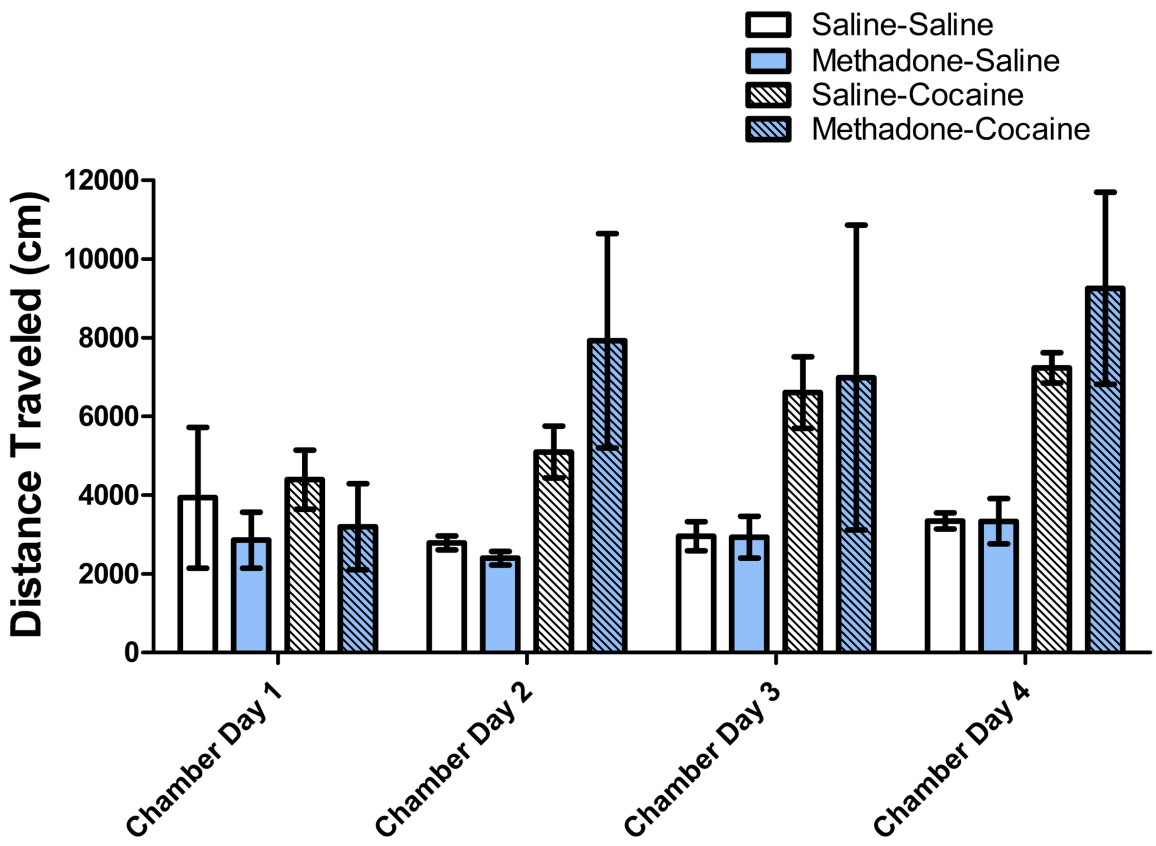

Figure 17. Average distance traveled for paired and unpaired mice across treatment groups throughout conditioning. A trend toward an interaction between time and cocaine revealed that paired animals showed greater activity compared to unpaired animals which progressively increased throughout conditioning and is consistent with context-specific sensitization (p = .06). There were no interactions between time and methadone (p = .313) or between time, methadone, and cocaine (p = .486) which suggests that methadone does not enhance cocaine sensitization (p = .742). Furthermore, there was no main effect of methadone (p = .742) or interaction between methadone and cocaine (p = .477) suggesting acute methadone does not affect cocaine-associated locomotion, independent of time.

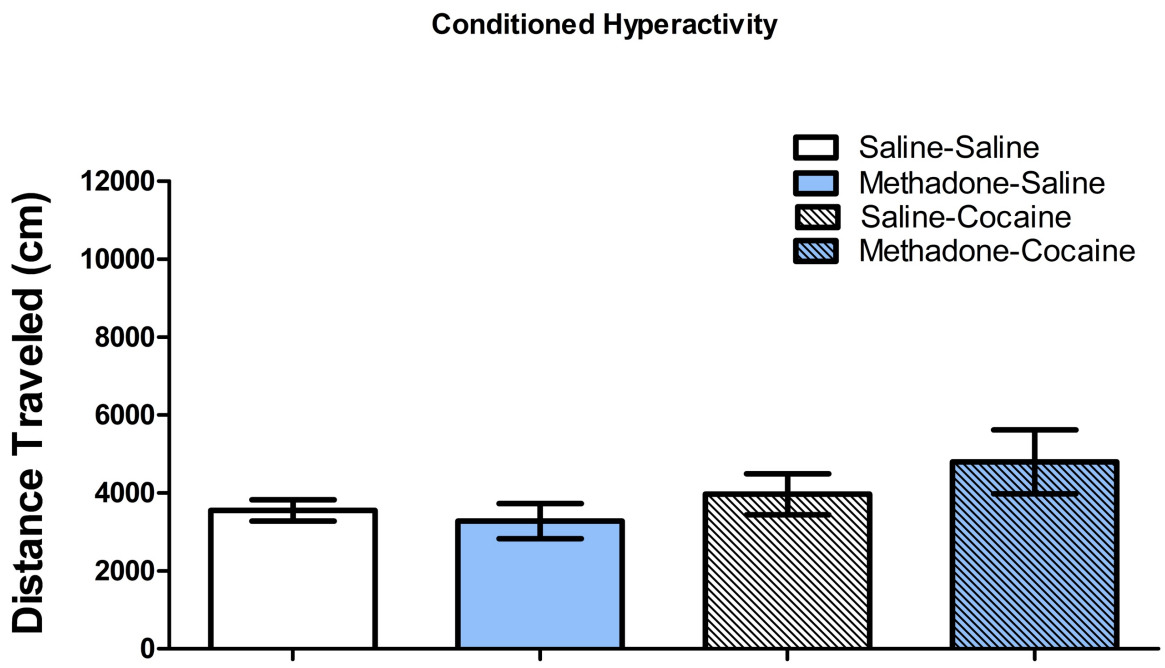

Figure 18. Average distance traveled for paired and unpaired mice when challenged with saline. A trend toward a main effect of Cocaine showed that paired mice were slightly more active and unpaired mice which is consistent with cocaine-conditioned hyperactivity ($p = .089$) but there was no main effect of Methadone ($p = .604$) or Methadone by Cocaine interaction ($p = .313$), suggesting that acute methadone administration does not affect cocaine-conditioned hyperactivity.

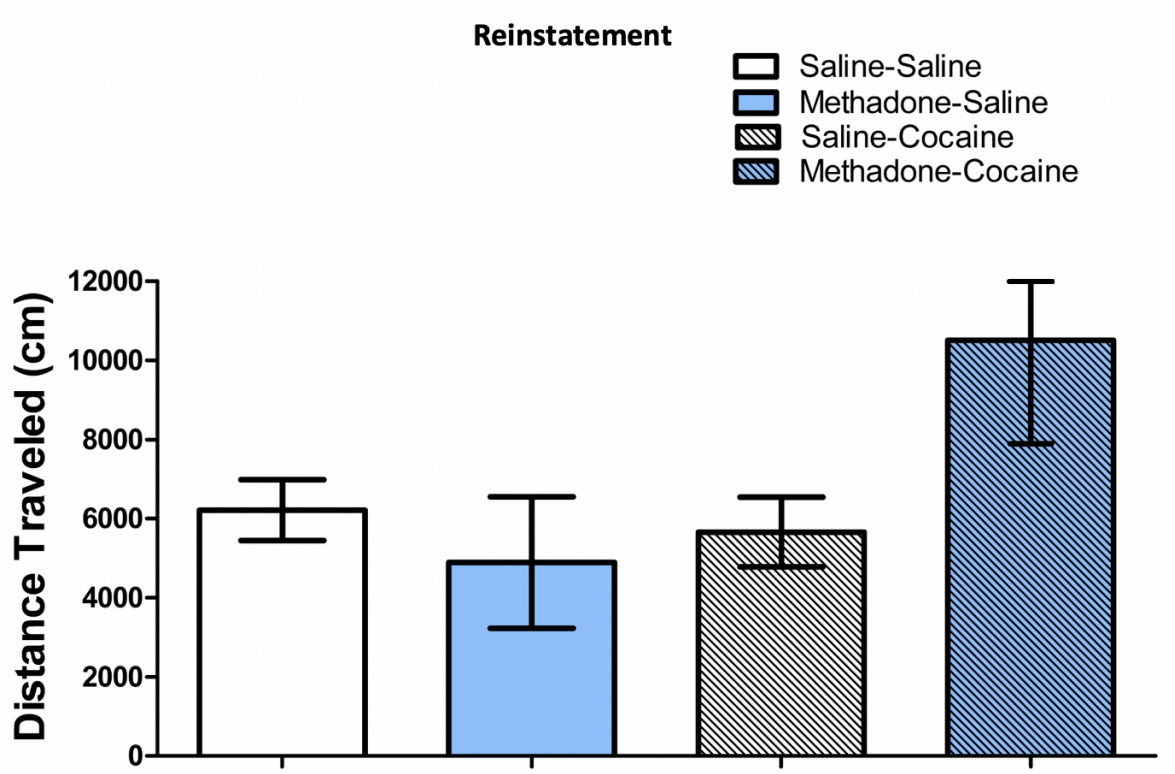

Figure 19. Average distance traveled for paired and unpaired mice when challenged with IP cocaine (10 mg/kg). A trend toward a main effect of cocaine showed that paired mice were more active compared to unpaired mice (p = .090) which is consistent with reinstatement. In addition, there was a statistically significant interaction between cocaine and methadone administration (p = .044) which suggests that acute methadone administration enhances cocaine-reinstated locomotor activity.

Discussion

Overview

Substance abuse and addiction have widespread health and social effects: they are associated with disease transmission (HIV, hepatitis), increased criminal activity, hospital admissions, and death (Anderson & Kearney, 2000). In particular, drug-associated deaths involving opioids such as heroin, oxycodone, morphine, and fentanyl, as well as stimulants such as cocaine and methamphetamine have been steadily increasing over the past decade (Hedegaard et al., 2018). Improved prevention methods and increased access to opioid use disorder treatment, including medication-assisted treatment, are needed to reduce opioid-associated morbidity and mortality.

Despite some drawbacks, methadone maintenance is associated with better patient retention and greater effectiveness compared to other pharmaceuticals such as buprenorphine and non-agonist treatments, and it remains the most well-studied and commonly used MAT drug (Veilleaux et al., 2010). However, treatment for opioid addiction can be complicated by polydrug use, and more specifically, the use of cocaine by patients in methadone maintenance programs (Leri et al., 2004). Research has shown that dopaminergic systems thought to underlie drug addiction and relapse can be co-regulated by cocaine and mu opioid receptor agonists, such as methadone. Furthermore, studies have revealed that these drugs may be acting in regions underlying drug-associated behaviors (sensitization) and memory (conditioned hyperactivity), such as the ventral tegmental area and the nucleus accumbens (Kaur, 2004; Steketee & Kalivas, 2011; Franklin & Druhan, 2000).

This overlap between the mechanism of action of cocaine and the endogenous opioid system has implications for polydrug use and suggests that mu opioid receptor activation might increase susceptibility to cocaine use. To date, there is no known research examining the effects of long-term methadone administration on cocaine conditioned hyperactivity and sensitization before the initial cocaine administration (and during subsequent exposures to cocaine). This study aimed to investigate the effect of chronic and acute methadone administration on cocaine associated behavior and memory using a sensitization and conditioned hyperactivity paradigm. The findings of the study indicate that chronic methadone may alter the magnitude of cocaine-induced locomotor activity but not cocaine sensitization, conditioned hyperactivity, or reinstatement. Meanwhile, acute methadone administration was found to enhance cocaine reinstatement. Together, these findings suggest that there remains a need to further evaluate the pharmacodynamics and pharmacokinetics of concomitant methadone and cocaine use, especially in regard to treating addiction and polydrug use.

Experiment 1: Chronic methadone administration does not affect the rate of cocaine-associated locomotor sensitization, conditioned hyperactivity, or reinstatement, but may enhance the stimulating effects of cocaine on locomotion

In order to examine the relationship between chronic methadone administration and cocaine locomotor sensitization and conditioned hyperactivity, mice received daily IP injections of low-dose methadone or saline (control) prior to undergoing cocaine conditioning. Throughout conditioning, paired mice received cocaine in the locomotor chamber, while unpaired mice received cocaine in their home cages. On chamber days, immediately after the cocaine or saline

injection, mice were allowed to freely roam throughout an open-field locomotor chamber and locomotor activity was assessed by distance traveled, and this alternated with days spent in the home cage. After conditioning, all mice were tested for conditioned hyperactivity by being challenged with saline. This hyperactivity test can also be perceived as an extinction period, where the conditioned stimulus (the locomotor chamber) no longer reliably predicts the unconditioned stimulus (cocaine). After the hyperactivity test, all mice were challenged with cocaine to test for reinstatement, which reflects susceptibility for relapse.

The findings of this study suggest that, contrary to the hypothesis, chronic methadone administration does not affect cocaine-associated sensitization, but interestingly, a trend suggests that chronic methadone may affect locomotion in cocaine-paired mice, independent of time. Throughout conditioning, paired animals overall were more active than unpaired animals and this locomotor activity increased over subsequent conditioning days, which is consistent with previous studies that have demonstrated cocaine-associated sensitization (Smith et al., 2016). However, chronic methadone does not appear to change the rate of acquisition of sensitization behavior. Instead, methadone-paired animals displayed enhanced locomotion beginning on the first day of cocaine exposure and this continued through the subsequent conditioning days to some plateau. Since methadone did not affect locomotor activity across the paired and unpaired groups, but only enhanced locomotion in the paired animals, this suggests that the trend was being driven by an interaction between methadone and cocaine and that methadone may be affecting the stimulatory effect of cocaine on locomotion.

Previous studies have examined the effect of methadone on cocaine use and received mixed results. Clinical research has reported that methadone may either increase, decrease, or

have no effect on cocaine use, however, these discrepancies may be caused by variations in methadone dosage (Leri et al., 2004). One study explored whether chronic methadone exposure modulated the direct stimulatory effects of acute cocaine in rats. Osmotic minipumps were implanted subcutaneously, and rats received various infusions of methadone which were within therapeutic range (0, 10, 20, or 30 mg/kg/day) before receiving an IP injection of cocaine (5 mg/kg). Locomotor activity tests were conducted to assess baseline, vehicle (saline), and cocaine activity, and the results indicated that chronic exposure to methadone elevated locomotor activity in a dose-dependent manner but did not enhance the acute stimulatory effects of cocaine on locomotion (Leri et al., 2004).

Meanwhile, the results of the conditioned hyperactivity test indicated that paired animals were significantly more active than unpaired animals, which reflects the cocaine-associated memory and conditioned response. These results are consistent with a wealth of studies that have used classical conditioning as a model of addiction to demonstrate a link between associative learning and drug abuse (Otto, Ocleirigh, & Pollack, 2007). After repeated pairings of a drug with a particular context or environment, the presentation of the conditioned stimulus (the drug-associated context or other cues) alone can elicit conditioned responses in rodents due to the formed association between the unconditioned stimulus (drug) and the previously neutral conditioned stimulus. This supports a current theory of addiction that hypothesizes that the formation of drug-associated memories can not only perpetuate drug-seeking behavior and drug use, but also can give rise to drug-seeking behavior following abstinence and this is thought to influence the long-term tendency to relapse (Milton & Everitt, 2012). However, the results in this study showed that there was no difference in locomotor activity in animals that received chronic

methadone vs. controls, and consistent with my predictions, these results suggest that chronic methadone does not affect the cocaine-associated memory.

The reinstatement test showed that paired mice were more active than unpaired mice. demonstrating reinstatement and suggesting that cocaine administration in a cocaine-associated context can lead to an increased susceptibility to relapse. This supports reports in the literature that individuals with a history of drug use show enhanced physiological responses to drug-associated cues compared with drug-naïve individuals, and this is prevalent across many drug classes (Milton & Everitt, 2012). In addition, patients' self-reported cocaine cravings in response to drug-associated cues correlated with changes in blood flow in the amygdala, anterior cingulate cortex (ACC) and basal ganglia in detoxified cocaine users compared with naïve counterparts, suggesting that drug use had changed the way these cues are processed (Childress et al., 1999). However, the results for the reinstatement test showed an absence of a methadone by cocaine interaction which suggests that chronic methadone does not affect reinstatement.

Experiment 2: Acute methadone administration enhances cocaine-induced reinstatement but not cocaine-associated locomotor sensitization or conditioned hyperactivity.

In order to determine whether the effects observed in the first experiment were attributed to the acute effects of methadone or the chronic administration regimen of the drug, I examined the effects of acute methadone administration on cocaine sensitization and conditioned hyperactivity. Mice received an IP injection of low-dose methadone or saline (control) 6 hours prior to undergoing cocaine conditioning. After conditioning, all mice were challenged with

saline to test for conditioned hyperactivity. On the following day, all mice were challenged with cocaine to test for reinstatement.

Similar to the first study, paired animals demonstrated greater locomotor activity compared to unpaired animals during cocaine conditioning, and this activity increased over time which is consistent with cocaine-associated sensitization. However, there were no methadone by cocaine or three-way interactions between methadone, time, and cocaine, and this suggests that acute methadone administration has no effect on cocaine sensitization or cocaine-associated locomotion, independent of time. Therefore, the synergistic effect on cocaine locomotion and the enhanced locomotion observed in methadone-paired mice compared to saline-paired mice in the previous experiment appears to be due to the chronic administration of the drug, but more research is needed to determine the nature of this effect.

In addition, the hyperactivity test results indicated that paired mice were slightly more active than unpaired mice, which is consistent with cocaine-conditioned hyperactivity, but similar to cocaine conditioning, there was no methadone by cocaine interaction which indicates that an acute dose of methadone does not affect cocaine associated memory as reflected by conditioned hyperactivity. Interestingly, the results from the reinstatement test revealed an interaction between cocaine and methadone. Methadone-paired mice showed enhanced activity reflected by increased distanced traveled, compared to saline-paired mice. Conversely, methadone-unpaired mice showed lower levels of locomotor activity compared to saline-unpaired mice. This suggests that acute methadone administration enhances cocaine-reinstated locomotor activity, a model of susceptibility to relapse.

This finding contradicts studies which have reported that methadone treatment may reduce cocaine use in heroin-cocaine users and that methadone administration was found to attenuate a variety of cocaine-motivated behaviors in rodents (Peles, Kreek, Kellogg, & Adelson, 2006; Leri, Zhou, Goddard, Cummins & Kreek, 2006). For instance, Leri et al. (2006) found that high-dose methadone maintenance via subcutaneous osmotic mini-pump reduced cocaine-induced reinstatement of cocaine-seeking behavior, cocaine self-administration on a progressive schedule of reinforcement, and cocaine place preference in rats. It was theorized that these effects were likely due to methadone's direct agonistic action at mu opioid receptors and consequent interactions with cocaine at neural sites controlling the expression of responses to cocaine and cocaine-associated stimuli, such as the nucleus accumbens and prefrontal cortex (Leri et al., 2006). However, this experiment differs from Leri et al. (2006) in that a low, acute dose of methadone was used (0.20 mg/kg), compared to steady-state administration of 20 and 55 mg/kg methadone, which suggests that the effects of methadone on cocaine-associated behaviors may be influenced by the dosage and the administration regimen.

Taken together, the results suggest that there may be a need to further investigate the pharmacodynamics and pharmacokinetics of concomitant methadone and cocaine administration, particularly with regard to developing more effective medication-assisted treatment methods for polydrug users.

Limitations and Future Directions

This study successfully produced preliminary behavioral data which are consistent with previous studies that have studied cocaine locomotor sensitization and conditioned hyperactivity

(Smith et al., 2016; Tilley et al., 2007). However, these experiments were underpowered and additional cohorts are needed to reach an optimal sample size as estimated by power analyses. For instance, only 16 mice completed the acute methadone study (Experiment 2) as two mice were excluded due to experimenter error. Of these mice, 5 were saline-paired, 3 were methadone-paired, 5 saline-unpaired, and 3 were methadone-unpaired. This contributed to a high amount of variability, especially in the methadone-paired animals, during each phase of the experiment (conditioning, hyperactivity test, and reinstatement test), which may bring into question the reliability of some of these the findings. Repeating these assays with larger sample sizes will increase statistical power and increase the probability of finding interactions or significant effects of methadone or cocaine, rather than trends.

A second limitation was that only male C57 mice were utilized in this study, so sex-based differences between male and females were left unexplored. NIDA reports that while men are more likely than women to use almost all types of illicit drugs and have higher rates of illicit drug and alcohol use and dependence, research has shown that women may use and respond to drugs differently, and may face obstacles to procuring treatment, such has being prescribed medication that is understudied in women. In addition, women may be more susceptible to key phases of addiction such as initiation of drug use, drug craving, and relapse (NIDA, 2018).

Some research suggests that polydrug use may be highly prevalent in women, which is associated with increased health risk. A recent study identified classes of polydrug use in a sample of women who used heroin, cocaine, and methamphetamine and explored the potential effect on risky sexual and drug use behaviors, as well as other factors, and concluded that the use of opioids and stimulants in three of the four polydrug use classes indicates that multi-modal

substance abuse therapies may be most effective in reducing drug-associated health risks among women (Lorvick et al., 2018).

Sex-based differences are similarly reported in rodent models of addiction. For example, one study found used cocaine conditioned place preference (CPP) to assess the subjective or rewarding effects of cocaine in male and female C57 mice. At low doses of cocaine, (2 mg/kg) results indicated that the acquisition of cocaine CPP did not differ between male and female mice, while males displayed delayed extinction compared to females (Hilderbrand & Lasek, 2014). Data suggest that the hormone estrogen may play a role in drug abuse in women, as periods of increased estrogen levels are associated with enhanced positive subjective responses following cocaine and amphetamine administration (Anker & Carrol, 2011). Similarly, studies have demonstrated that female rats in estrus were more susceptible to reinstatement, displaying heightened sensitivity to the motivational and stimulant effects of cocaine (Kippen et al., 2005). This is further supported by animal studies which have shown that the administration of estrogen can facilitate cocaine acquisition and reinstatement (Anker & Carroll, 2011). Given that patterns of drug use and response to drugs of abuse may differ between men and women and that many of these differences are reflected in rodent models of addiction, future cohorts in this study should include both female and male mice to assess whether the effects of methadone administration on cocaine-associated behavior and memory observed in this study differ by sex.

In addition, it should be noted that the sensitization and conditioned hyperactivity paradigm is not the only method that can be used to study opioid and cocaine-associated behavior and memory. For instance, in our behavioral paradigm, cocaine reinstatement (which reflects the susceptibility for relapse) was assessed after a single day of extinction (the conditioned

hyperactivity test). Other studies have used longer periods of extinction in which rodents are placed into a previously drug-associated environment, but responses are not reinforced by drug delivery. However, the duration of this period is highly variable among different studies (Kufahl & Olive, 2011).

In addition, other behavioral assays such as the conditioned place preference (CPP) may provide more sensitive assessments of memory and drug-seeking behavior, since this paradigm requires rodents to choose between a drug-associated environment and a non-associated environment, such as cocaine. The conditioned place preference (CPP) assay is commonly used to study drug-associated memories and to measure the rewarding or aversive effects of drugs, and through classical conditioning, it allows for the assessment of reward-related learning and reflects the drug-associated memory (Smith et al., 2016). A future study could assess the effect of methadone administration on cocaine-associated behavior and memory in a manner similar to the CPP and extinction paradigm utilized by Malvaez et al. (2013).

Animals could be assigned to methadone-cocaine, saline-cocaine, methadone-saline, and saline-saline groups and undergo either acute or chronic methadone administration. After a pretest to assess baseline preference for the cocaine-paired chamber (i.e. checkered) compared to an unpaired chamber (i.e. white), mice would undergo cocaine conditioning, where mice are exposed to cocaine or saline in one of the two environments for a limited time. These pairings are complemented by pairings of the same length conducted in the opposite chamber on alternating days. Conditioning can then be followed by a period of extinction to assess the strength of the drug memory, as animals with a stronger memory would be expected to spend more time in the checkered chamber compared to the white chamber. Then, in a test for

reinstatement, all animals would be given cocaine and allowed to explore the chambers (Fig. 20). It is expected that animals with a stronger cocaine memory would reinstate higher (have a greater preference for the checkered chamber compared to the white chamber).

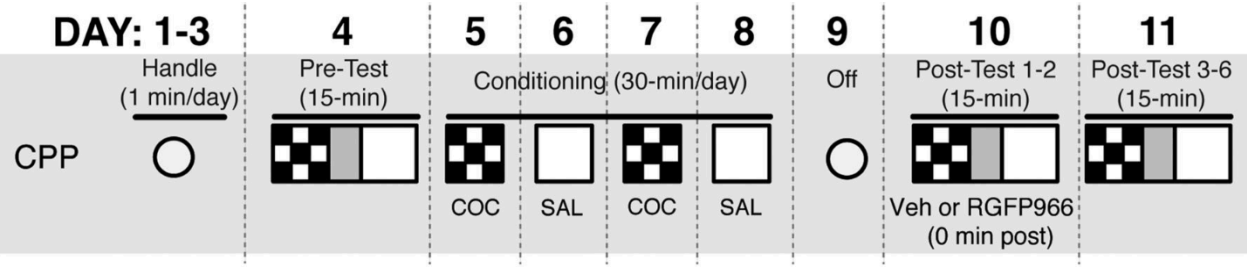

Figure 20. Cocaine conditioned place preference and extinction paradigm. Adapted from Malvaez et al., (2013).

The results of the current study suggest that chronic methadone may be interacting with cocaine to affect cocaine's stimulating effect on locomotor activity and that acute methadone may increase cocaine sensitization, but the exact nature of this interaction is unknown. Therefore, future work should utilize molecular techniques to look for regulatory changes in regions implicated in conditioned hyperactivity (nucleus accumbens) or sensitization (ventral tegmental area) and that are thought to underlie drug addiction (Rogge & Wood, 2012). Brains could be harvested either after the first day of cocaine exposure (after chronic methadone administration) or after reinstatement (after acute methadone administration). After harvesting, tissue punch can be used to look at changes in gene transcription or translation using techniques such as mRNA/RT-PCR or Western blot. Other directions of interest include looking for

epigenetic markers, such as histone acetylation via histone acetyltransferases (HATS) or histone deacetylases (HDACs), for genes that are regulated by cocaine such as *Fos*, *Fosb*, *Bdnf II*, and *Cdk5* (Rogge & Wood, 2012; Malvaez et al., 2013). In particular, specific HDACs appear to be involved in the extinction of drug-seeking behavior (Malvaez et al., 2013).

Conclusion

Substance abuse disorder is characterized by compulsive drug-seeking behavior regardless of negative consequences. Methadone, a synthetic opioid, has been used as an effective treatment to aid rehabilitating patients in reducing illicit opioid use and maintaining abstinence. The concurrent use of cocaine during medication-assisted treatment (MAT) is highly prevalent and may complicate opioid recovery. Both methadone and cocaine act on the reward pathway of the brain to produce distinct and complementary changes in drug-associated behaviors and memories. This study investigated the effect of chronic and acute methadone administration on cocaine-conditioned hyperactivity and sensitization in mice. Results indicated that chronic methadone does not affect the rate of cocaine sensitization, conditioned hyperactivity, or reinstatement. However, a trend suggests that chronic methadone may enhance the locomotor activating effect of cocaine. Similarly, acute methadone was found to have no effect on cocaine sensitization or conditioned hyperactivity, but an interaction revealed that acute methadone enhanced cocaine reinstatement, which reflects susceptibility for relapse. These results suggest that further research is needed to elucidate the effects of concomitant methadone and cocaine use and this could have important implications for the development of improved medication-assisted treatment methods for polydrug use.

Appendix

Testing Group	Animal # Meth/Sal	Pairing	Weight (g)	Dose (mL)	Meth/Sal IN Carr 126	Chamber Day IN TESTING ROOM	Chamber #	Home Cage Day IN Carr 126
Group 1	1	Paired	35.4 g	0.35 mL	Saline	cocaine	1	saline
	2	Unpaired	38.8 g	0.39 mL	Saline	saline	2	cocaine
	3	P	34.1 g	0.34 mL	Meth	cocaine	3	saline
	4	U	30.4 g	0.30 mL	Meth	saline	4	cocaine
Group 2	5	P	30.6 g	0.31 mL	Sal	cocaine	2	saline
	6	U	37.6 g	0.38 mL	Sal	saline	3	cocaine
	7	P	36.0 g	0.36 mL	Meth	cocaine	4	saline
	8	U	42.0 g	0.42 mL	Meth	saline	1	cocaine
Group 3	9	P	38.2 g	0.38 mL	Sal	cocaine	3	saline
	10	U	33.2 g	0.33 mL	Sal	saline	4	cocaine
	11	P	35.6 g	0.36 mL	Meth	cocaine	1	saline
	12	U	40.3 g	0.40 mL	Meth	saline	2	cocaine
Group 4	13	P	37.7 g	0.38 mL	Sal	cocaine	4	saline
	14	U	36.8 g	0.37 mL	Sal	saline	1	cocaine
	15	P	34.1 g	0.34 mL	Meth	cocaine	2	saline
	16	U	35.5 g	0.36 mL	Meth	saline	3	cocaine
Group 5	17	P	35.8 g	0.36 mL	Sal	cocaine	1	saline
	18	U	36.2 g	0.36 mL	Sal	saline	2	cocaine

Group 1	19	Paired	24.6	0.25	Saline	cocaine	1	saline
	20	Unpaired	25.2	0.25	Saline	saline	2	cocaine
	21	P	23.9	0.24	Meth	cocaine	3	saline
	22	U	24.3	0.24	Meth	saline	4	cocaine
Group 2	23	P	26.0	0.26	Sal	cocaine	2	saline
	24	U	25.7	0.26	Sal	saline	3	cocaine
	25	P	26.5	0.27	Meth	cocaine	4	saline
	26	U	24.2	0.24	Meth	saline	1	cocaine
Group 3	27	P	23.6	0.24	Sal	cocaine	3	saline
	28	U	24.6	0.25	Sal	saline	4	cocaine
	29	P	26.7	0.27	Meth	cocaine	1	saline
	30	U	24.9	0.25	Meth	saline	2	cocaine
Group 4	31	P	27.8	0.28	Sal	cocaine	4	saline
	32	U	28.2	0.28	Sal	saline	1	cocaine
	33	P	26.5	0.27	Meth	cocaine	2	saline
	34	U	25.4	0.25	Meth	saline	3	cocaine
Group 5	35	P	24.2	0.24	Sal	cocaine	1	saline
	36	U	24.5	0.25	Sal	saline	2	cocaine

Table 3. Conditioning treatments for Experiment 1 group assignments. Mice were divided into Paired (n= 9) or Unpaired (n= 9) groups, represented by the green and blue cells, respectively. Paired mice (blue) receive cocaine in locomotor chamber, while unpaired mice (green) receive cocaine in the home cage. Among the paired and unpaired animals, there are some mice receiving chronic methadone (purple; n total = 16), and the rest receiving chronic saline (white; n total = 10). These group assignments are consequently reflected in the conditioning treatments on Chamber and Home Cage days.

Day 1 (1/2)	Day 2 (1/3)	Day 3 (1/4)	Day 4 (1/5)	Day 5 (1/6)	Day 6 (1/7)	Day 7 (1/8)
Inject 0.2 mg/kg methadone (n = 8) or vehicle (n=10) Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg Methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126
Day 8 (1/9)	Day 9 (1/10)	Day 10 (1/11)	Day 11 (1/12)	Day 12 (1/13) Handle	Day 13 (Tue 1/14) Handle and Habituate	Day 14 (1/15) Handle and Habituate
Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126	Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Handle 2 min Testing Room	Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs, Handle 2 min Testing Room Place mice in locomotor box and record activity for 30 minutes	Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs, Handle 2 min Testing Room Place mice in locomotor box and record activity for 30 minutes
Conditioning Day 15 (1/16)	Conditioning Day 16 (1/17)	Conditioning Day 17 (1/18)	Conditioning Day 18 (Saturday 1/19)	Conditioning Day 19 (Sunday 1/20)	Conditioning Day 20 (Monday 1/21)	Conditioning Day 21 (Tuesday 1/22)
CHAMBER 7:00 AM Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs (1:00 PM) In Testing Room **Paired (n=9):** Cocaine injection (10 mg/kg) **Unpaired (n=9):** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	CHAMBER Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	CHAMBER Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	CHAMBER Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber
Conditioning Day 22 (Wed 1/23)	Test Day 23 Hyperactivity (Thurs 1/24)	Test Day 24 Reinstatement (Friday 1/25)	Data Analysis Day 25 (1/26)			
HOME CAGE Inject 0.2 mg/kg methadone or vehicle Carr 126 After 6 hrs Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	Administer vehicle injection to all mice Testing Room place in locomotor activity chamber for 30 min and record	Administer Cocaine injection to all mice Testing Room Place in locomotor activity chamber for 30 min and record				

Table 4. Experiment schedule to investigate the effects of chronic methadone on cocaine hyperactivity and sensitization. Mice are assigned to Paired and Unpaired groups, with mice receiving an IP injection of acute methadone (0.2 mg/kg) or acute vehicle in each group. To establish a chronic methadone administration regimen, mice receive a daily IP methadone or saline injection, depending on group assignment (Day 1-22). Mice will be handled for 2 min/day during a 3-day handling period (Day 12-14), followed by habituation in locomotor chambers (Day 13-14). Conditioning will occur over eight alternating Chamber and Home Cage days (Day 15-22). Six hours after the daily methadone (0.20 mg/kg) or saline injection, Paired mice receive an IP injection of cocaine (10 mg/kg) and Unpaired mice receive saline, with activity recording for 30 minutes. On Home Cage days, Paired mice will be given an IP injection of saline, while Unpaired mice received cocaine (10 mg/kg). In the Hyperactivity test, all mice will be challenged with vehicle, placed in the locomotor box, and recorded for 30 minutes. At the Reinstatement test, all mice were challenged with cocaine (10 mg/kg), placed in the locomotor box, and recorded for 30 minutes.

Testing Group	Animal # Meth/Sal	Pairing	Weight (g)	Dose (mL)	Meth/Sal IN Carr 126	Chamber Day IN TESTING ROOM	Chamber #	Home Cage Day IN Carr 126
Group 1	1	Paired	28.0	0.28	Saline	cocaine	1	saline
	2	Unpaired	31.4	0.31	Saline	saline	2	cocaine
	3	P	27.0	0.27	Meth	cocaine	3	saline
	4	U	29.4	0.29	Meth	saline	4	cocaine
Group 2	5	P	31.7	0.32	Sal	cocaine	2	saline
	6	U	28.7	0.29	Sal	saline	3	cocaine
	7	P	32.6	0.33	Meth	cocaine	4	saline
	8	U	30.4	0.30	Meth	saline	1	cocaine
Group 3	9	P	30.3	0.30	Sal	cocaine	3	saline
	10	U	30.6	0.31	Sal	saline	4	cocaine
	11	P	30.8	0.31	Meth	cocaine	1	saline
	12	U	28.2	0.28	Meth	saline	2	cocaine
Group 4	13	P	32.2	0.32	Sal	cocaine	4	saline
	14	U	30.6	0.31	Sal	saline	1	cocaine
	15	P	34.0	0.34	Meth	cocaine	2	saline
	16	U	30.4	0.30	Meth	saline	3	cocaine
Group 5	17	P	26.7	0.27	Sal	cocaine	1	saline
	18	U	31.2	0.31	Sal	saline	2	cocaine

Table 5. Conditioning treatments for Experiment 2 group assignments. Mice were divided into Paired (n= 9) or Unpaired (n= 9) groups, represented by the yellow and orange cells, respectively. Paired mice (yellow) receive cocaine in locomotor chamber, while Unpaired mice (orange) receive cocaine in home cage. Among the Paired and Unpaired animals, there are 4 mice receiving chronic methadone (purple; n total = 8), and 5 receiving chronic saline (white; n total = 10). These group assignments are consequently reflected in the conditioning treatments on Chamber and Home Cage days.

Day 1 Handling	Day 2 Handling + Habituation	Day 3 Handling + Habituation	Conditioning Day 4	Conditioning Day 5	Conditioning Day 6	Conditioning Day 7
Handle 2 min Testing Room Weigh all mice and prepare drug solutions	Handle 2 min Testing Room Place mice in locomotor box and record activity for 30 minutes	Handle 2 min Testing Room Place mice in locomotor box and record activity for 30 minutes	CHAMBER Inject 0.2 mg/kg meth or vehicle (8:30) Carr 126 After 6 hrs Testing Room **Paired:** Cocaine (10 mg/kg) **Unpaired:** Vehicle Record activity for 30 min	HOME CAGE Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	CHAMBER Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages
Conditioning Day 8	**Conditioning Day 9**	**Conditioning Day 10**	**Conditioning Day 11**	**Hyperactivity Test Day 12**	**Reinstatement Test Day 13**	**Data Analysis Day 14**
CHAMBER Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	CHAMBER Testing Room **Paired Mice:** Cocaine injection (10 mg/kg) **Unpaired:** Vehicle injection Record activity for 30 min in locomotor chamber	HOME CAGE Carr 126 **Paired mice:** Vehicle **Unpaired:** Cocaine injection (10 mg/kg) Place mice back in home cages	Administer vehicle injection to all mice Testing Room place in locomotor activity chamber for 30 min and record	Administer Cocaine injection to all mice (10 mg/kg) Testing Room Place in locomotor activity chamber for 30 min and record	

Table 6. Experiment schedule to investigate the effects of acute methadone on cocaine hyperactivity and sensitization. Mice are assigned to Paired and Unpaired groups, with mice receiving an IP injection of acute methadone (0.2 mg/kg) or acute vehicle in each group. Mice were handled for 2 min/day during a 3-day handling period (Day 1-3), followed by habituation in locomotor chambers (Day 2-3). Cocaine conditioning occurred over eight alternating Chamber and Home Cage days (Day 4-11). On the first chamber day (Day 4), mice received an acute IP injection of methadone (0.20 mg/kg) or saline. Six hours later, Paired mice received an IP injection of cocaine (10 mg/kg) and Unpaired mice received saline, with activity recording for 30 minutes. The following Chamber days followed the same procedure as the first, but without the acute methadone injection. On Home Cage days, Paired mice received an IP injection of saline, while Unpaired mice received cocaine (10 mg/kg). In the Hyperactivity test, all mice were challenged with vehicle, placed in the locomotor box, and recorded for 30 minutes. At the Reinstatement test, all mice were challenged with cocaine (10 mg/kg), placed in the locomotor box, and recorded for 30 minutes.

Literature Cited

Al-Hasani, R., & Bruchas, M. R. (2011). Molecular Mechanisms of Opioid Receptor-dependent Signaling and Behavior. *Anesthesiology,* 1363-1381.

Anderson, I. B., & Kearney, T. E. (2000). Use of methadone. *Western Journal of Medicine*, *172*(1), 43–46.

Anker, J. J., & Carroll, M. E. (2011). Females are more vulnerable to drug abuse than males: Evidence from preclinical studies and the role of ovarian hormones. In J. C. Neill & J. Kulkarni (Eds.), *Biological basis of sex differences in psychopharmacology.* (Vol. 8, pp. 73–96). New York, NY: Springer-Verlag Publishing.

Bart, G. (2012). Maintenance Medication for Opiate Addiction: The Foundation of Recovery. *Journal of Addictive Diseases*, *31*(3), 207–225.

Beyer, C.E., & Steketee, J. D. (2002). Cocaine Sensitization: Modulation by Dopamine D_2 Receptors, *Cerebral Cortex*, 12(5), 526–535.

Brecht, M. L., Huang, D., Evans, E., & Hser, H. I. (2008). Polydrug use and implications for longitudinal research: ten-year trajectories for heroin, cocaine, and methamphetamine users. *Drug and Alcohol Dependence*, *96*(3), 193–201.

Burton, A. C., Bissonette, G. B., Vazquez, D., Blume, E. M., Donnelly, M., Heatley, K. C., Hinduja, A., & Roesch, M. R. (2018). Previous cocaine self-administration disrupts reward expectancy encoding in ventral striatum. *Neuropsychopharmacology, 43*(12), 2350–2360.

Calipari, E. S., Ferris, M.J., Zimmer, B.A., Roberts, D.C., & Jones, S.R. (2013). Temporal pattern of cocaine intake determines tolerance vs sensitization of cocaine effects at the dopamine transporter. *Neuropsychopharmacology, 38*(12), 2385–2392.

Center for Disease Control and Prevention. (2018, December 19). Opioid overdose. Retrieved from https://www.cdc.gov/drugoverdose/epidemic/index.html

Childress, A. R., Mozley, P. D., McElgin, W., Fitzgerald, J., Reivich, M., & O'Brien, C. P. (1999). Limbic activation during cue-induced cocaine craving. *American Journal of Psychiatry*, (1), 11.

Contet, C., Kieffer, B. L., & Befort, K. (2004). Mu opioid receptor: a gateway to drug addiction. *Current Opinion in Neurobiology*, *14*(3), 370–378.

Erdo, F., Polgar, K., Mate, I., & Szekely, J. (1990). Modulation of dopamine-induced hypermotility following injection of various opioids into the nucleus-accumbens. *Acta Physiologica Hungarica, 76*(3), 219–227.

Fields, H. L., & Margolis, E. B. (2015). Understanding opioid reward. *Trends in Neurosciences,38*(4), 217-225.

Franklin, T. R., & Druhan, J. P. (2000). Involvement of the Nucleus Accumbens and Medial Prefrontal Cortex in the Expression of Conditioned Hyperactivity to a Cocaine-Associated Environment in Rats. *Neuropsychopharmacology,23*(6), 633-644.

Grueter, B. A., Robison, A. J., Neve, R. L., Nestler, E. J., & Malenka, R. C. (2013). ΔFosB differentially modulates nucleus accumbens direct and indirect pathway function. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(5), 1923–1928.

Grissinger M. (2011). Keeping patients safe from methadone overdoses. *P & T : a peer-reviewed journal for formulary management*, *36*(8), 462-6.

Hedegaard, H., Bastion, B., Trinidad, J.P., Spencer, M., & Warner, M. (2018) Drugs most frequently involved in drug overdose deaths: United States, 2011-2016. *National Vital Statistics Reports*, *67*(9), 1-13.

Hilderbrand, E. R., & Lasek, A. W. (2014). Sex differences in cocaine conditioned place preference in C57BL/6J mice. *NeuroReport, 25*(2), 105-109.

Holuj, M., Bisaga, A., & Popik, P. (2013). Conditioned rewarding effects of morphine and methadone in mice pre-exposed to cocaine. *Pharmacological Reports*, *65*, 1176–1184.

Hyman, S. E. (2005). Addiction: A Disease of Learning and Memory. *American Journal of Psychiatry, 162*(8), 1414-1422.

Kauer, J. (2004). Learning mechanisms in addiction: Synaptic plasticity in the ventral tegmental area as a result of exposure to drugs of abuse. *Annual Review of Physiology 66*, 447–475.

Kippin, T. E., Fuchs, R. A., Mehta, R. H., Case, J. M., Parker, M. P., Bimonte-Nelson, H. A., & See, R. E. (2005). Potentiation of cocaine-primed reinstatement of drug seeking in female rats during estrus. *Psychopharmacology, 182*(2), 245-252.

Kreek, M. J., Levran, O., Reed, B., Schlussman, S. D., Yan, Z., & Butelman, E. R. (2012). Opiate addiction and cocaine addiction: underlying molecular neurobiology and genetics. *Journal of Clinical Investigation*, (10), 3387.

Kufahl, P. R., & Olive, M. F. (2011). Investigating Methamphetamine Craving Using the Extinction-Reinstatement Model in the Rat. *Journal of addiction research & therapy*, *S1*(3), 003.

Leri, F., Bruneau, J., & Stewart, J. (2003). Understanding polydrug use: review of heroin and cocaine co-use. *Addiction*, *98*(1), 7.

Leri, F., Tremblay, A., Sorge, R., & Stewart, J. (2004). Methadone maintenance reduces heroin- and cocaine-induced relapse without affecting stress-induced relapse in a rodent model of poly-drug use. *Neuropsychopharmacology* 29(7), 1312-1320.

Leri, F., Zhou, Y., Goddard, B., Cummins, E., & Kreek, M. J. (2006). Effects of High-Dose Methadone Maintenance on Cocaine Place Conditioning, Cocaine Self-Administration, and Mu-Opioid Receptor mRNA Expression in the Rat Brain. *Neuropsychopharmacology*, *31*(7), 1462–1474.

Leri, F., Zhou, Y., Carmichael, B., Cummins, E., & Kreek, M. J. (2012). Treatment-like steady-state methadone in rats interferes with incubation of cocaine sensitization and associated alterations in gene expression. *European Neuropsychopharmacology*, *22,* 143-152.

Lorvick, J., Browne, E. N., Lambdin, B. H., & Comfort, M. (2018). Polydrug use patterns, risk behavior and unmet healthcare need in a community-based sample of women who use cocaine, heroin or methamphetamine. *Addictive Behaviors, 85*, 94-99.

Lynch, W. J., Nicholson, K. L., Dance, M. E., Morgan, R. W., & Foley, P. L. (2010). Animal models of substance abuse and addiction: implications for science, animal welfare, and society. *Comparative medicine*, *60*(3), 177-88.

Malvaez, M., Mcquown, S. C., Rogge, G. A., Astarabadi, M., Jacques, V., Carreiro, S., . . . Wood, M. A. (2013). HDAC3-selective inhibitor enhances extinction of cocaine-seeking behavior in a persistent manner. *Proceedings of the National Academy of Sciences, 110*(7), 2647-2652.

Mattick RP, Breen C, Kimber J, & Davoli M. (2009) Methadone maintenance therapy versus no opioid replacement therapy for opioid dependence. *Cochrane Database of Systematic Reviews, 3*, CD002209.

Mattick, R. P., Breen, C., Kimber, J., & Davoli, M. (2014). Buprenorphine maintenance versus placebo or methadone maintenance for opioid dependence. *Cochrane Database of Systematic Reviews* 2, CD002207.

McCance-Katz, E. F., Jatlow, P., & Rainey, P. M. (2010). Effect of Cocaine Use on Methadone Pharmacokinetics in Humans. *American Journal on Addictions*, *19*(1), 47–52.

Mckim, W. A. (2007). *Drugs and behavior: An introduction to behavioral pharmacology* (6th ed.). Upper Saddle River, NJ: Prentice Hall.

Meyer, J. S., & Quenzer, L. F. (2005). *Psychopharmacology: Drugs, the brain and behavior*. Sunderland, MA: Sinauer Associates.

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

Morrison, S. E., McGinty, V. B., Hoffmann, J. D., & Nicola, S. M. (2017). Limbic-motor integration by neural excitations and inhibitions in the nucleus accumbens. *Journal of Neurophysiology, 118*(5), 2549-2567.

National Institute on Drug Abuse. (2018, July 12). Substance Use in Women. Retrieved from https://www.drugabuse.gov/publications/research-reports/substance-use-in-women on 2019, April 24.

National Institute on Drug Abuse. (2018, January 17). Principles of Drug Addiction Treatment: A Research-Based Guide (Third Edition). Retrieved from https://www.drugabuse.gov/publications/principles-drug-addiction-treatment-research-based-guide-third-edition on 2018, June 8.

National Institute on Drug Abuse. (2018, July). Cocaine. Retrieved from https://www.drugabuse.gov/publications/drugfacts/cocaine.

National Institute on Drug Abuse. (2018). Drugs and the Brain. Retrieved from https://www.drugabuse.gov/publications/drugs-brains-behavior-science-addiction/drugs-brain.

National Institute on Drug Abuse. (2018). Is drug addiction treatment worth its cost? Retrieved from https://www.drugabuse.gov/publications/principles-drug-addiction-treatment-research-based-guide-third-edition/frequently-asked-questions/drug-addiction-treatment-worth-its-cost.

Nestler E. J. (2005). The neurobiology of cocaine addiction. *Science & practice perspectives*, *3*(1), 4-10.

Oliva, E. M., Maisel, N. C., Gordon, A. J., & Harris, A. H. S. (n.d.). Barriers to Use of Pharmacotherapy for Addiction Disorders and How to Overcome Them. *Current Psychiatry Reports*, *13*(5), 374–381.

Ostlund, S. B., & Cui, Y. (2018). Not worth the wait: cocaine alters reward processing in the nucleus accumbens. *Neuropsychopharmacology: Official Publication Of The American College Of Neuropsychopharmacology*, *43*(12), 2333–2334.

Otto, M., Ocleirigh, C., & Pollack, M. (2007). Attending to Emotional Cues for Drug Abuse: Bridging the Gap Between Clinic and Home Behaviors. *Science & Practice Perspectives, 3*(2), 48-56.

Peles, E., Kreek, M. J., Kellogg, S., Adelson, M. (2006). High methadone dose significantly reduces cocaine use in methadone maintenance treatment (MMT) patients. *J Addict Dis*. 2006;25(1):43–50.

Reed, B., Butelman, E. R., & Kreek, M. J. (2017). Endogenous opioid system in addiction and addiction-related behaviors. *Current Opinion in Behavioral Sciences*, *13*, 196–202.

Robinson, T. E., & Kolb, B. (2004). Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology*, *47*, 33–46.

Rogge, G. A., & Wood, M. A. (2012). The role of histone acetylation in cocaine-induced neural plasticity and behavior. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *38*(1), 94-110.

Schreiber, S., Barak, Y., Hostovsky, A., Baratz-Goldstein, R., Volis, I., & Pick, C. G. (2014). Interaction of Different Antidepressants with Acute and Chronic Methadone in Mice, and Possible Clinical Implications. *Journal of Molecular Neuroscience*, *52*(4), 598-604.

Serdarevic, M., Striley, C. W., & Cottler, L. B. (2017). Gender differences in prescription opioid use. *Current opinion in psychiatry*, *30*(4), 238-246.

Smith, L. N., Penrod, R. D., Taniguchi, M., & Cowan, C. W. (2016). Assessment of cocaine-induced behavioral sensitization and conditioned place preference in mice. *Jove-Journal Of Visualized Experiments*, (108), e53107.

Spanagel, R., Herz, A., & Shippenberg, T. (1990). Identification of the opioid receptor types mediating beta-endorphin-induced alterations in dopamine release in the nucleus-accumbens. *European Journal of Pharmacology*, *190*(1–2), 177–184.

Spangler, R., Zhou, Y., Maggos, C., Zlobin, A., Ho, A., & Kreek, M. (1997). Dopamine antagonist and “binge” cocaine effects on rat opioid and dopamine transporter mRNAs. *Neuroreport*, *7*(13), 2196–2200.

Steketee, J. D., & Kalivas, P. W. (2011). Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. *Pharmacological reviews*, *63*(2), 348–365.

Substance Abuse and Mental Health Services Administration. (2017) Key substance use and mental health indicators in the United States: results from the 2016 National Survey on Drug Use and Health. HHS publication no. SMA 17–5044, NSDUH Series H-52. Rockville, MD: Center for Behavioral Health Statistics and Quality, Substance Abuse and Mental Health Services Administration.

Tilley, M. R., Cagniard, B., Zhuang, X., Han, D. D., Tiao, N., & Gu, H. H. (2007). Cocaine reward and locomotion stimulation in mice with reduced dopamine transporter expression. *BMC Neuroscience*, *8*(42).

US Food and Drug Administration. (2018, May 22). Information by Drug Class - Information about Medication-Assisted Treatment (MAT). Retrieved from https://www.fda.gov/Drugs/DrugSafety/InformationbyDrugClass/ucm600092.htm on 2018, June 13.

Vivolo-Kantor, A. M., Seth, P., Gladden, R. M., Mattson, C. L., Baldwin, G. T., Kite-Powell, A., & Coletta, M. A. (2018). Vital Signs: Trends in Emergency Department Visits for Suspected Opioid Overdoses--United States, July 2016-September 2017. *Morbidity and Mortality Weekly Report*, p. 279.

Wenzel, J. M., Rauscher, N. A., Cheer, J. F., & Oleson, E. B. (2014). A role for phasic dopamine release within the nucleus accumbens in encoding aversion: a review of the neurochemical literature. *ACS chemical neuroscience*, *6*(1), 16–26.

White, A. O., & Rauhut, A. S. (2014). Time-dependent effects of prazosin on the development of methamphetamine conditioned hyperactivity and context-specific sensitization in mice. *Behavioral Brain Research*, 263, 80-9.

Zhang, Y., Zhu, X., Huang, C., & Zhang, X. (2015). Molecular changes in the medial prefrontal cortex and nucleus accumbens are associated with blocking the behavioral sensitization to cocaine. *Scientific reports*, *5*, 16172.