THE ROLE OF THE PATCH COMPARTMENT NEURONS IN METHAMPHETAMINE-MEDIATED REWARD

By

TROY KENDRICK

B.S., Biology, Wake Forest University 2016

A Thesis Submitted to the Faculty

of Mercer University of Medicine

in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN BIOMEDICAL SCIENCES

Macon, GA

2018

THE ROLE OF PATCH COMPARTMENT NEURONS IN METHAMPHETAMINE MEDIATED REWARD

By

TROY KENDRICK

Approved:

_____ Date_____
Ashley Horner, Ph.D.
Faculty Advisor for Thesis, Thesis Committee Member

_____ Date _____
Susan Cline, Ph.D.
Thesis Committee Member

_____ Date _____
Richard McCann, Ph.D.
Program Director, Thesis Committee Member

_____ Date _____
Jean Sumner, MD
Dean, Mercer University School of Medicine

TABLE OF CONTENTS

ABSTRACT

THE ROLE OF PATCH COMPARTMENT NEURONS IN METHAMPHETAMINE MEDIATED REWARD

By TROY KENDRICK

Under the direction of KRISTEN HORNER, Ph.D.

The striatum is significantly important in the formation of habitual behaviors as well as reward association. Elicited reward behavior from methamphetamine (METH) is mediated by the striatum. Two distinct striatal sub regions, the patch and matrix, have been shown by previous studies to have key relationship in the regulation of addiction behavior. The patch compartment, in contrast with the matrix, expresses a high density of mu opioid receptors and receives dense inputs from limbic regions of the brain. Patch compartment neurons contribute to habitual behaviors, and our hypothesis is that these neurons also contribute to reward. The goal of this work was to investigate the role of the patch compartment neurons in METH-induced reward behavior. The approach was designed to determine if the ablation of mu opioid receptor-containing neurons of the patch compartment would alter METH-mediated Condition Placed Preference (CPP). This was achieved by using Dermorphin-Saporin (DERM-SAP), a neurotoxin that specifically targets and eliminates mu opioid receptor-containing neurons. Male and female rats in this study were bilaterally infused in striatum either with DERM-SAP or unconjugated Saporin (SAP), which was used as a control. After eight days of recovery,

subjects were placed into the CPP paradigm, receiving either a moderate dose of methamphetamine (2mg/kg) or saline. After eight days of conditioning, a preference test was conducted on each animal. It was found that METH-mediated CPP was reduced in female rats which were pretreated with DERM-SAP, while METH-mediated CPP was increased in male rats with DERM-SAP lesions. These results allow us to conclude that patch compartment neurons are necessary for METH-induced reward behaviors in females, but not males.

INTRODUCTION

With violent drug crimes and overdose rates soaring to new heights, addiction has become a stifling problem and a rampant disease in modern society. According to the National Institute of Drug Abuse, more than 64,000 Americans died from drug overdose in 2016. The drug methamphetamine (METH) is responsible for more than ten percent of these deaths. As METH has become a leading killer in some communities, and with addiction exponentially progressing, a clear need exists for a more finite understanding of neurobiological reward pathways. Enhanced knowledge of reward pathways would allow for proper drug treatment and prevention. Viable drug treatment therapy for METH addiction would not only diminish drug overdose rates, but also would reduce the nearly 700-billion-dollars that the United States spends annually on substance abuse. Detailed research on METH reward pathways and drug seeking behaviors will be key in the eradication of addiction. While directly studying the habitual aspect of drug abuse will be helpful in forming therapies, proper insight on drug seeking and reward behaviors within the basal ganglia will lead to targeted cures for those suffering from a seemingly incurable disease.

Methamphetamine is a highly abused central nervous system stimulant with high reward properties that lead to compulsive drug seeking behavior (Park, Shen, Tien, Roman, & Ma, 2011). Alterations in the levels of dopamine within the synapse work to elicit the feelings of euphoria that accompany METH consumption. METH readily crosses the blood-brain barrier and has the ability to enter nerve terminals by passing directly through nerve cell membranes, where it induces the release of neurotransmitter within the synapse (National Institutes of Health, 2018). By blunting the effects of monoamine oxidase, METH is able to block the breakdown of excess dopamine within

the synapse. Prolonged excess dopamine levels lead to desensitization of dopamine receptors, inevitably leading to an increase in the frequency and amount of METH use. The physiological actions of METH are mediated by GABAergic medium spiny efferent neurons of the striatum. METH-induced increases in extracellular dopamine alters the function of these neurons, which ultimately influences behavior (Garrett & Holtzman, 1996; Guix, Hurd, & Ungerstedt, 1992; Wise, 1996) . Medium spiny efferent striatal neurons are distributed across two neurochemically and anatomically distinct compartments: the patch and matrix (Gerfen, 1984; Graybiel, 1990; Parent, Cicchetti, & Beach, 1995). A better understanding of how METH reward and drug seeking behaviors flow through these sub-regions of the striatum will allow the mediation of behavioral effects of drug abuse.

Addiction is defined as a chronic, relapsing brain disease characterized by compulsive drug seeking and use, despite harmful consequences (NIDA, 2014). "Hard-wired" changes in the brain are considered critical for the transition from casual to addictive drug use (Adinoff, 2004). The drastic differences in short term METH use and long-term METH abuse serve as a prime example. Short term METH use causes euphoria, hyperactivity, and hyperthermia, while long term METH abuse can result in extreme dependence, addiction, and psychosis. Previous METH administration studies have shown that animals remain sensitized for many weeks, suggesting that the development of sensitization involves long-lasting neuronal adaptations. These neural alterations underlie behavioral sensitization as well as compulsive drug seeking behavior (Park et al., 2011). While the initial rewarding aspects of drug taking may be goal directed, once habitual behaviors are established, the behavior continues in response to

the stimulus, without concern for the value of the outcome (Canales, 2005; Chersi & Pezzulo, 2012; Zapata, Minney, & Shippenberg, 2010). With that being said, drug seeking is pathological and inflexible and therefore may be more closely related with habitual behaviors (Ostlund & Balleine, 2008).

Habit learning processes have been implicated in the transition from recreational drug use to the compulsive drug seeking that characterizes addiction (Everitt et al., 2008; Everitt & Robbins, 2005; White, 1996). The primary stages of drug taking are thought to be goal directed and controlled by the action outcome contingency (Adams & Dickinson, 1981; Balleine & Dickinson, 1998), while continued drug consumption leads to habitual behavior that has increasing involvement with stimulus response (S-R) (Dickinson, 1985; Zapata et al., 2010). The striatum has been implicated in aspects of motivational and learning processes that support goal directed (action outcome) behavior, as well as reward seeking and habitual behavioral that concur with stimulus response association (Balleine & Dickinson, 1998; Kravitz & Kreitzer, 2012). Circuit links that project from the cortex to the striatum play a central role in developing appropriate response behaviors (Haber, 2016). A heterogenous mosaic of neurochemically and functionally distinct neurons, the striatum has distinct regions with differing functions (Gerfen, 1984; Graybiel, 1990; Haber, 2016). Network connectivity between the nucleus accumbens (NAc) prefrontal cortex (PFC) and dorsomedial striatum (DMS) plays a role in the acquisition of goal directed actions (Barker, Taylor, & Chandler, 2014; Coutureau & Killcross, 2003). The dorsolateral striatum (DLS) is highly innervated by the sensory motor cortices (SMC) and is responsible for habitual behavior and reward seeking behavior (SR) (McGeorge & Faull, 1989; Pan, Mao, & Dudman, 2010; Yin & Knowlton,

2006). The DMS and DLS receive divergent, but overlapping inputs, from cortical and subcortical structures as well as from some specialized limbic projections that are associated with reward (Barker et al., 2014; Canales, 2005).

Previous work has suggested that the patch compartment of the striatum mediates the reward process, given that animals repeatedly self-stimulate if an electrode is placed within the patch compartment (White & Hiroi, 1998). The patch compartment is anatomically connected through re-entrant loops with limbic prefrontal and allocortical structures, which play a part in stimulant induced reinforcement (Canales, 2005). Neurons of the patch give rise to one-third of GABAergic striatal output pathway innervating dopamine neurons in the substantia nigra pars compacta, which is rich in dopaminergic neurons (Gerfen, 1984; Jimenez-Castellanos & Graybiel, 1987). Pathways which innervate the patch compartment of the striatum are critically positioned to influence the way in which dopamine and drugs affect dopaminergic neurotransmission and regulate basal ganglia output (Canales & Graybiel, 2000). Recent preliminary data from our laboratory shows that the patch compartment is necessary for reward processes, showing that decreased activation of the patch compartment neurons diminishes the rewarding effects of METH (Horner, Logan, Fisher, & Logue, 2017).

Neurobiological differences in the patch and matrix compartment of the striatum are likely to be responsible for their differing functions. The patch compartment, in contrast with the matrix, has a high density of mu opioid receptors. Previous studies have reported that mu opioid receptor knockout mice were resistant to METH-induced behavioral sensitization, proving that mu opioid receptors are pivotal in the reward association process (A. Becker et al., 2002). As fore noted previous studies suggest that

limbic reward based basal ganglia pathways are centered in the patch compartment system and that nodal points for sensorimotor processing are anchored primarily within the matrix compartment (Canales, 2005; Jimenez-Castellanos & Graybiel, 1987). The research of Brown and Colleagues, which involved metabolic studies on the neutral behavior of rats, found that the neuronal activity in the matrix compartment peaked during normal ongoing behavior and during the processing of sensory input. Analysis of these data indicated that during this metabolic increase in the matrix compartment, there was no engagement of reward seeking behaviors (Brown et al., 2002). Findings of Brown and colleagues, along with the electrode patch self-stimulation studies of White and Hiroi 1998, work to show that the patch neurons are primarily the target of phasic reward related signals conveyed by dopaminergic projections and corticostriatal fibers (Canales, 2005).

The patch and matrix compartments are arranged in a mosaic throughout the striatum (Gerfen, 1984). It is believed that limbic projections of the patch compartment are thought to be influential in the sensorimotor networks in the DLS that mediate habit formation. Previous studies have provided direct evidence that the dorsolateral striatum is necessary for habit formation. Furthermore, when the habit system is disrupted, control over instrumental performance reverts to the system controlling the performance of goal-directed instrumental actions (PFC-DMS) (Yin, Knowlton, & Balleine, 2004). Recent studies, as well as prior research from our lab, have suggested that enhanced activation of the patch compartment relative to the matrix compartment are observed following psychostimulant exposure (Canales & Graybiel, 2000; Horner et al., 2017). With this information, we propose that enhanced activation of the patch compartment of the DLS

pathway could be responsible for METH reward and drug seeking behaviors, given their inflexibilities. Initially, the patch compartment of the DMS (action outcome) contributes to reinforcing aspects of METH, underlying the association between drug and environment. Continued METH use will result in the enhancement of the patch compartment of the DLS (Stimulus Response), mediating inflexible behaviors such as drug seeking.

Previous studies have suggested that the development of habitual responding reflects active inhibition of goal directed responses that are mediated by action outcome association (Coutureau & Killcross, 2003). It is thought that the patch compartment, when preferentially activated, might modulate loops that traverse the DLS leading to enhanced habitual and inflexible behaviors. The absence of the patch compartment not only leads to devaluation of reward but also loss of modulation between flexible and inflexible behaviors, causing the DMS circuits to come back online. It is unclear how dopaminergic inputs stretching from the substantia nigra pars compacta to the DMS and DLS differ, or if enhanced activation of the patch compartment of either of these striatal regions participates in drug-related reward. Our study set out to investigate the role of patch compartment neurons in METH-induced reward and drug seeking behaviors. This research consisted of an in depth analysis of the DLS neuronal activity after METH-mediated condition placement preference (CPP) of rats in which the mu opioid receptor neurons in the patch compartment had been chemically ablated. Using a METH-mediated CPP paradigm, we were able to determine that the patch compartment of the DLS contributes to reward and drug seeking behaviors. The use of CPP provides valuable

input on the inflexible behaviors of drug seeking and is a standard preclinical behavioral model for researching reward.

The compiled research based on the projected circuits of the patch and matrix compartments suggest that enhanced activation of neuronal activity in the patch compartment of the DLS may be necessary for the transition from normal drug use to the inflexible drug seeking and reward behaviors seen in addiction. We hypothesize that METH-mediated CPP will preferentially increase activation in the SMC-DLS stimulus response loop (habitual behavior) and that ablation of the patch compartment will change this behavior to a preferential increase in the PFC-DMS action outcome loop (goal directed).

METHODS and MATERIALS

Drugs:

(±) Methamphetamine hydrochloride was provided by the National Institute on Drug Abuse (Bethesda, MD, USA). Ketamine hydrochloride and xylazine hydrochloride used for anesthesia were obtained from Sigma Aldrich (St. Louis, MO, USA). All drugs were given in a volume of 1 ml/kg and dissolved in normal saline after doses were calculated as free base conversion. Dermorphin-saporin (DERM-SAP) and unconjugated saporin (SAP) was obtained from Advanced Targeting Systems (San Diego, CA, USA). Both were dissolved in artificial cerebrospinal fluid (aCSF; 144 mM NaCl; 2.68 mM KCl; 1.6 mM $CaCl_2$; 2.6 mM $MgCl_2$; 0.4 mM KH_2PO_4, pH, 7.2).

Animals:

Male and female Sprague-Dawley rats were used for the experiment, males weighing between 250-400 g, and females weighing between 200-300 g. All rats were house breed in Mercer University School of Medicine animal care facility. Approximately three weeks after birth rats were weaned from their mothers and placed inside plastic cages in same sex groups of three or four. Rats were housed within a temperature-controlled room on a 14:10 hour light/dark cycle, and given free access to food and water. All animal care and experimental manipulations were approved by the Institutional Animal Care and Use Committee (IACUC) of Mercer University School of Medicine and were in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Direct measures were taken to minimize any suffering that might've occurred during experimentation.

Surgeries:

At five to six weeks of age rats underwent bilateral infusion surgery. Rats were anesthetized using ketamine (90 mg/kg) and xylazine (9 mg/kg) using an intraperitoneal injection. Rats where then fixed into a stereotaxic frame (Stoelting Company, Wood Dale, IL, USA) to be held into position during surgery. Once fixed into position the skull of the rat was exposed, and a hole was drilled on the right and left side of the rat's skull. A 29-guage needle was then carefully placed into each hole in order to access the striatum. The needles were allowed to sit for one minute before 2 µl of DERM-SAP or SAP (vehicle) was infused bilaterally into the striatum. The coordinates for proper infusion are based on bregma (+1.7 mm anterior, +2.6 mm lateral, -5.0 mm ventral: (Paxinos and Watson) and drugs were infused at a rate of 0.5 µl/min. Needles were left in place for one minute after the infusion and slowly removed to minimize fluid backflow. Animals were then stapled up with wound clips and returned to their home cages where they were allowed eight days to recover. Only animals with successful infusions in the rostral striatum were included for detailed analyses.

CPP:

Conditioning chambers for this experiment were constructed using plexiglass. Each chamber was divided into two sections, one half of the chamber painted a solid gray, while the other sided painted with black and white stripes 2.5 cm in width. Box patterned stainless steel flooring was used on the striped section of each chamber, and circular patterned stainless steel was used for the flooring of the solid gray section. This distinct flooring was added as a tactical que for each rat when conditioning. Separate conditioning chambers were constructed for males and females to eliminate variation. A

removable partition divided the chamber one sided painted gray, the other striped. Rats were habituated to the chamber for one day before the conditioning procedure by being placed in the open chamber with no partition for 30 minutes. 24 hours later the rat was then placed in the open chamber without the partition for 20 minutes and recorded for bias testing. This footage was immediately analyzed using the software Anymaze to determine the rats initial bias for the striped or solid side. The following day conditioning began with eight alternating days of METH or saline injections. Rats were confined to either their preferred side or drug side based upon Anymaze analysis of bias testing. On conditioning days 1, 3, 5, and 7 rats were weighed and given a subcutaneous dose of saline equal to their body weight (g/ml), prior to being placed on their preferred side of the conditioning chamber for 30 minutes. On conditioning 2, 4, 6, and 8 rats were weighted and given a subcutaneous dose of Meth equal to their body weight (2 mg/kg, g/ml), prior to being placed on their nonpreferred or drug side of the chamber for 30 minutes. 24 hours after the end of conditioning rats were placed into open chambers for preference testing, each rat was recorded for 20 minutes and immediately sacrificed. Preference test footage was then analyzed by Anymaze to determine time spent on each side of the chamber by the rat. The difference in seconds between the time spent on the drug side (nonpreferred side) post conditioning and the drug side (nonpreferred side) pre-conditioning, allows a preference score to be determined. A positive preference score indicates conditioned place preference, while a negative preference score indicated aversion.

Estrous Tracking:

Estrous tracking was performed in order to observe variation in results between male and female rats throughout experimentation. Keeping a strict timeline of the female rat cycle allowed for a rough overview of particular hormone surges within the rat which could possibly affect behavioral results. Vaginal cells respond keenly to circulating levels of hormones within the rat. Observation of vaginal cell morphology allows a noninvasive technique for determining whether the rat is in proestrus, estrus, metestrus, or diestrus (Goldman, Murr, & Cooper, 2007). Given that estrous synchronization takes place by acclimating rats to particular light cycles and male rats within the room, only one female from each home cage was tested during experimentation (Harris & Kesel, 1990). Each day just prior to CPP, a fresh cotton swab was wet with DI water and placed inside the vaginal canal of each tested female. A single clockwise rotation of the cotton swab was used to collect vaginal cells, and then the swab was streaked across a glass slide. Vaginal smears were allowed to air dry for one minute and stored at -20° C. For microscopic evaluation, vaginal smears were allowed to thaw for one hour then observed under a vista view microscope at 4X magnification. A clear image of the microscope view for each separate slide was captured using a video camera, and digital images were used to classify the phase of the estrous cycle. Proestrus was classified by nucleated epithelial cells, estrus was classified as 75% nucleated cells and 25% cornified cells, metestrus was classified as abundant leukocytes with nucleated and cornified cells, while diestrus was classified by the presence of only leukocytes (Lohmiller & Swing, 2006). Characterization of vaginal smears were performed without awareness of treatment groups. However, for accuracy purposes blind assessment of vaginal cytology was not

used, patterns were tracked in the cycle of individual animals to monitor transition between days and provide a more exact depiction for cycle status each day (Goldman et al., 2007). *Figure 1* was used as guide in classifying vaginal cytology.

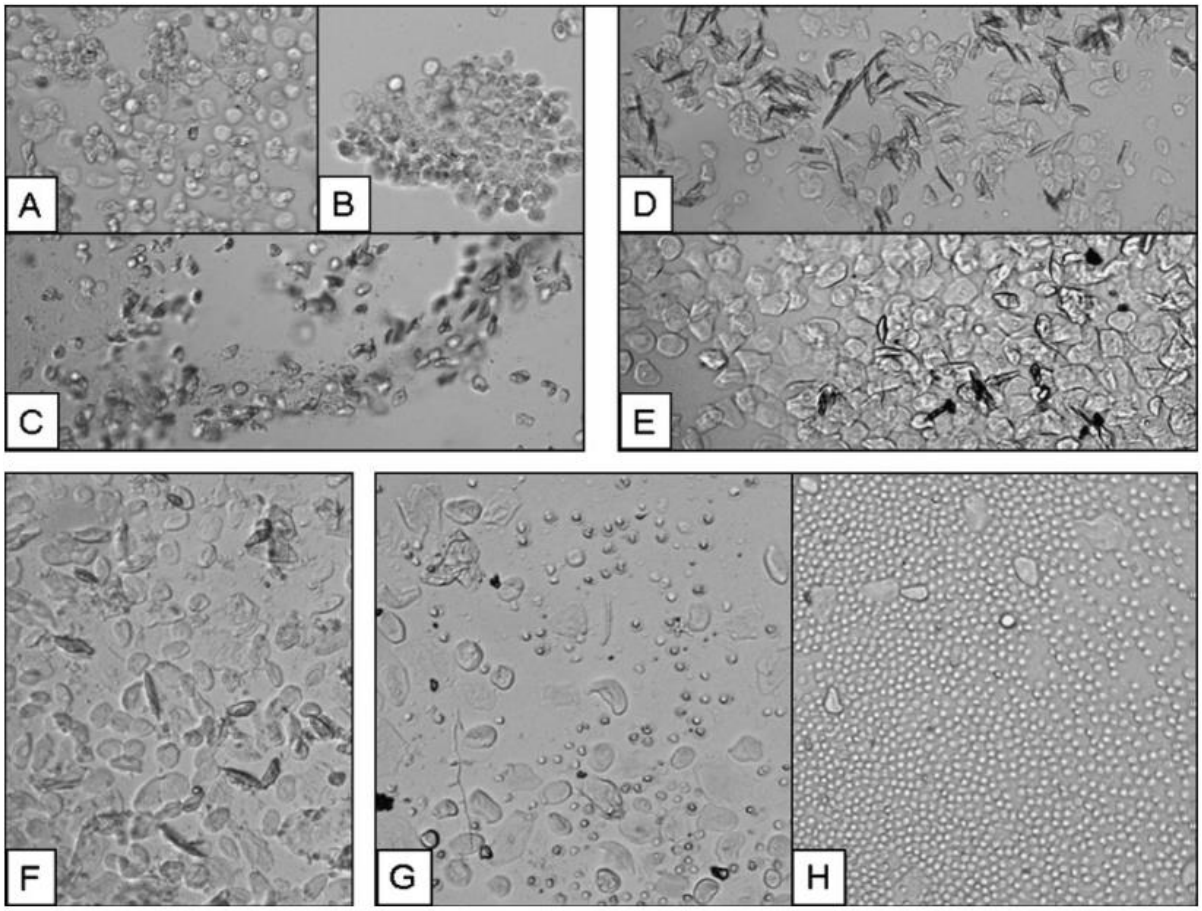

Figure 1: Representative wet, unstained vaginal smears obtained on different days of the rat (Long-Evans hooded strain) estrous cycle. A,B: Proestrus; cells tend to appear in clumps and have a granular appearance. C: Proestrus; cells can alternatively be present as strands. D: Estrus; classic keratinized, needle-like cells. E: Estrus; cells can alteratively appear rounded, with jagged irregular edges. F: Metestrus; a combination of round ‘‘pavement cells,’’ some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. G: Diestrus; leukocytes can appear in combination with various larger rounded cells. H: Diestrus; classic leukocytic smear with a few larger round epithelial cells.(Goldman et al., 2007)

Tissue Processing:

Directly after experimentation rats were sacrificed via exposure to CO_2 and then decapitated. Rat brains were then rapidly harvested and flash frozen in isopentane on dry ice. Brains were stored at -80° C until they were cut using a cryostat (Minotome Plus, Triangle Biomedical Sciences, Durham, NC, USA) into 12-µm sections through the frontal cortex, striatum, thalamus, and substantia nigra. Processed sections were placed on glass slides and held at -20° C until ready for immunohistochemistry.

C-Fos Immunohistochemistry:

Sections from tissue processing were allowed to thaw and then outlined using a pap pen. Slides were then rinsed three times in PBS buffer, each wash lasting five minutes. Tissue was then fixed in 4% paraformaldehyde for five minutes at 37° C, and then rinsed in PBS buffer three times. A blocking solution made up of 4% normal horse serum (NHS), 4% dry milk powder, 0.3% triton-x-100, PBS, and avidin block was applied to each slide and allowed to sit at room temperature for 1 hour inside a humidity chamber. Directly after slides were allowed to incubate at 4° C overnight with a rabbit primary antibody for c-Fos diluted 1:1000 in 0.3% triton-x-100 PBS solution. The next day the slides were washed three times in PBS and incubated two hours with mixture of biotinylated horse-anti-rabbit IgG antiserum (Vector laboratories), NHS, and PBS at room temperature. Slides were then rinsed three times in PBS incubated for 1 hour in ABC solution (Elite ABC kit, Vector Laboratories), and again rinsed three times in PBS. Slides were then covered in DAB- Nickel solution for approximately 2-4 minutes which detected bound antibody. After staining slides were washed in deionized water and

dehydrated in a series of alcohol rinses. Lastly slides were cover slipped out of xylene and allowed to dry for further image analysis.

Image analysis:

C-Fos labeled sections from each rat were captured using a VistaVision microscope (VWR, Radnor, PA, USA) at magnification 4X with an attached video camera (CCD Moticam 2300, Motic, Richmond, BC, Canada). The computer imaging program Image J was used to outline a 400x400 region of dense mu opioid receptor immunoreactivity for each section (DMS, DLS, PFC, SMC). The number of c-Fos labeled particles that exceeded the threshold of density for each region analyzed was determined using the particle analysis option in Image J. For accurate analysis, prior to thresholding a pixel range for particle seize was determined by outlining approximately 15-20 positively labeled cells from 10-15 randomly selected sections. This technique was used to determine the average size of labeled cells in terms of pixel area. The lower limit for a properly labeled c-Fos cell on the particle analysis setting was set to the smallest number of pixels measured for any cell, and the upper limit was set at the maximal particle size on the particle analysis of Image J. Background staining was eliminated and immunoreactive pixels for the selected regions were measured. Counts of c-Fos labeled particles were expressed as the number of c-Fos immunoreactive particles per mm^2.

Statistical analysis:

Using Prism statistical analysis software (Prism 4. GraphPad Software, Inc. 5755 Oberlin Drive, #110 San Diego, CA 9212), a two-way ANOVA test was performed to determine the correlation between the effects of DERM-SAP pretreatment and METH

treatment in the number of labeled c-Fos particles within the medial striatum, lateral striatum, prefrontal cortex, and sensorimotor cortex. A post-hoc Bonferonni analysis was used to compare all four groups (Vehicle/METH, Vehicle/Saline, DERM-SAP/METH, DERM-SAP/Saline) and determine significant difference between each group.

RESULTS

The effects of DERM-SAP pretreatment on METH-mediated CPP:

METH-mediated CPP along with the targeted degradation of mu opioid receptors within the patch compartment of the striatum using DERM-SAP, was used in order to determine whether the patch compartment of the striatum is necessary for METH-induced reward. Preference scores were generated for each rat by calculating the difference in the amount of time spent in the drug-paired side of the chamber post conditioning and the amount of time spent in the initially non-preferred side of the chamber. A two-way ANOVA analysis on these preference scores indicated that in male rats, METH treatment was able to significantly induce CPP in both DERM-SAP lesioned and non-lesioned animals (P=0.0018) (*Figure 2*). DERM-SAP pretreatment in male rats had no main significance in attributing to CPP aversion (P=0.4257). In female rats, a significant main effect of DERM-SAP pretreatment (P=0.0130) was observed. In contrast to males, a significant interaction between DERM-SAP pretreatment and METH treatment was noted in female rats (P=0.0183) (*Figure 3*). Female rats lesioned with DERM-SAP showed a reduced METH-induced CPP. Given the significance between DERM-SAP METH treated animals and Sap METH treated animals, we can conclude that ablation of the mu opioid receptors of the patch compartments in female rats reduces METH-induced CPP.

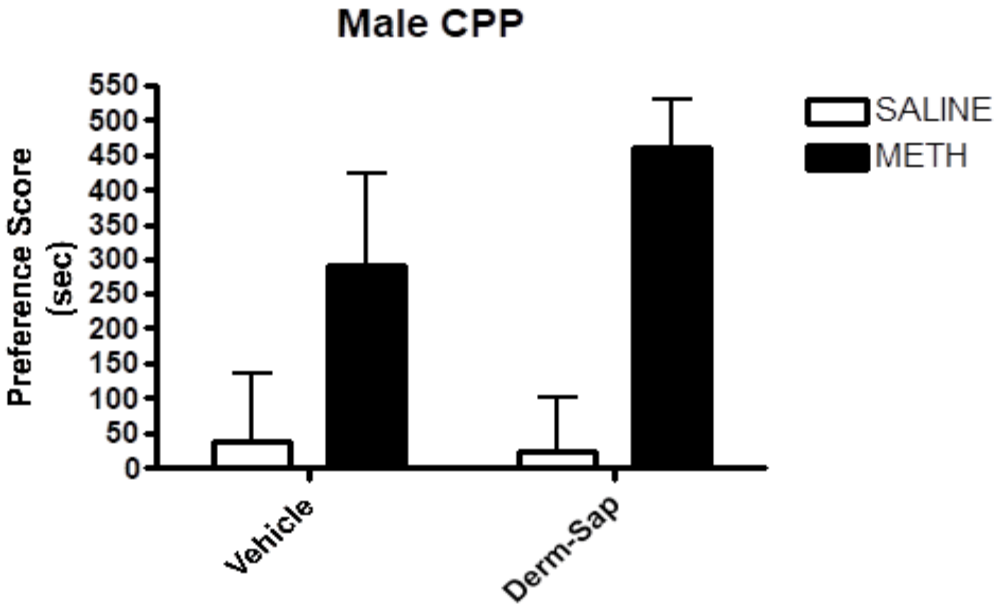

Figure 2: Effects of bilateral intracranial infusion of DERM-SAP on METH-mediated CPP in male rats. Preference score values are calculated from the number of seconds spent in the drug-paired side of the chamber (initially non-preferred) post conditioning, minus the time spent on the non-preferred side of the chamber pre-conditioning. Ablation of the patch compartment neurons of the striatum in male rats did not reduce METH-induced CPP. METH was able to significantly induce CPP in DERM-SAP treated males as well as vehicle treated males (SAP).

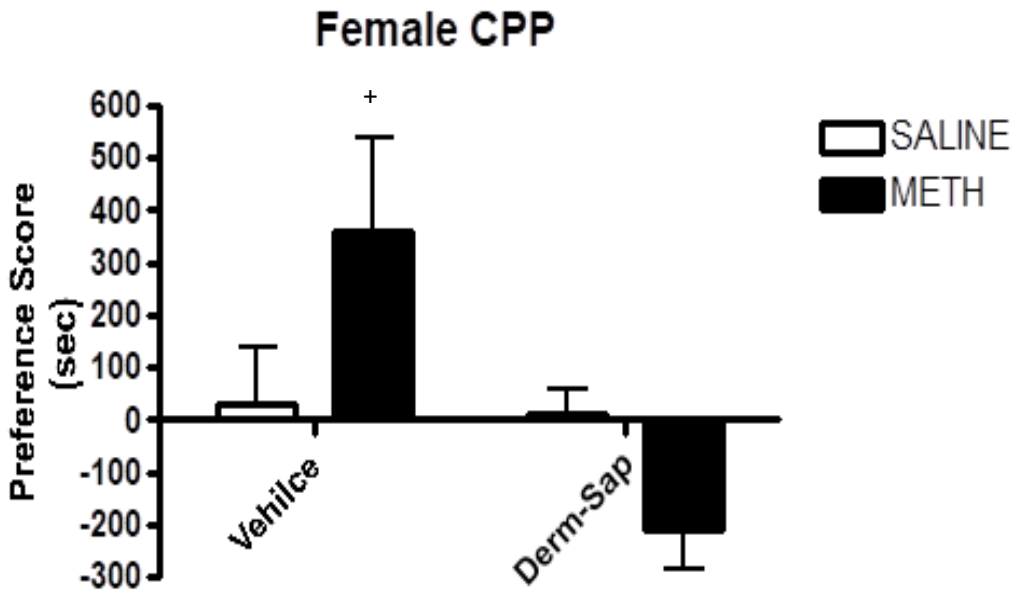

Figure 3: Effects of bilateral intracranial infusion of DERM-SAP on METH-mediated CPP in female rats. Preference score values are calculated from the number of seconds spent in the drug-paired side of the chamber (initially non-preferred) post conditioning, minus the time spent on the non-preferred side of the chamber pre-conditioning. Ablation of the patch compartment neurons of the striatum in female rats reduced METH-induced CPP. There was a significant interaction between DERM-SAP and METH. +=$p<0.05$ vs. respective saline-treated group.

Effects of DERM-SAP Pretreatment on C-Fos Immunoreactivity in the Prefrontal Cortex:

C-Fos immunoreactivity is a direct way to measure neuronal activity within particular compartments of the brain. After performing a quantitative analysis of the c-Fos particles of the prefrontal cortex of female rats, a two way ANOVA analysis was run. This analysis of c-Fos immunoreactivity revealed that there was a significant difference between vehicle METH treated animals and vehicle saline treated animals (P=0.0417) (*Figure 4*). Further post-hoc analysis revealed that there was no significant difference in the prefrontal cortex of female DERM-SAP METH treated animals, and DERM-SAP saline treated animals (P=0.2735). The analysis of c-Fos levels within the prefrontal cortex of female rats showed that DERM-SAP pretreatment was successful in preventing METH-induced c-Fos increases within the prefrontal cortex.

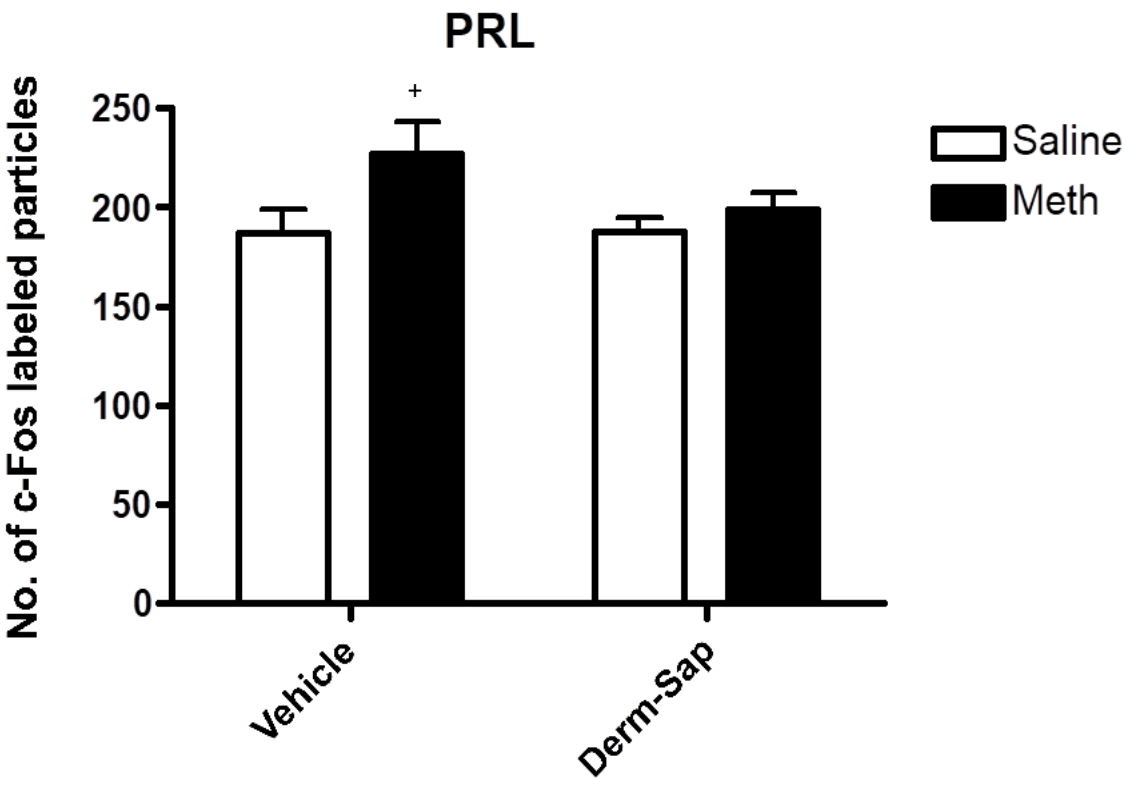

Figure 4: Effects of DERM-SAP pretreatment on c-Fos expression within the prefrontal cortex. Quantitative results were gathered from a 400 x 400 pixel area in the prefrontal cortex of all female rats. Analysis shows that there is a significant difference in the number of c-Fos particles between Vehicle/Saline and Vehicle/METH, but not a significant difference in the number of c-Fos particles between DERM-SAP/Saline and DERM-SAP/METH in PFC. DERM-SAP prevented METH-induced increase in c-Fos expression in the PFC. +=$p<0.05$ vs. respective saline-treated group.

Effects of DERM-SAP Pretreatment on C-Fos Immunoreactivity in the Dorsomedial Striatum:

After performing a quantitative analysis of the c-Fos particles of the dorsomedial striatum of female rats, a two way ANOVA analysis was run. This analysis of c-Fos immunoreactivity revealed that there was a significant difference between vehicle METH treated animals, and vehicle saline treated animals (P=0.0096) (*Figure 5*). Further post-hoc analysis revealed that there was no significant difference in the dorsomedial striatum of female DERM-SAP METH treated animals, and DERM-SAP saline animals (P=0.0598). The analysis of c-Fos levels within the dorsomedial striatum of female rats showed that DERM-SAP pretreatment was successful in preventing METH-induced c-Fos increases within the dorsomedial striatum.

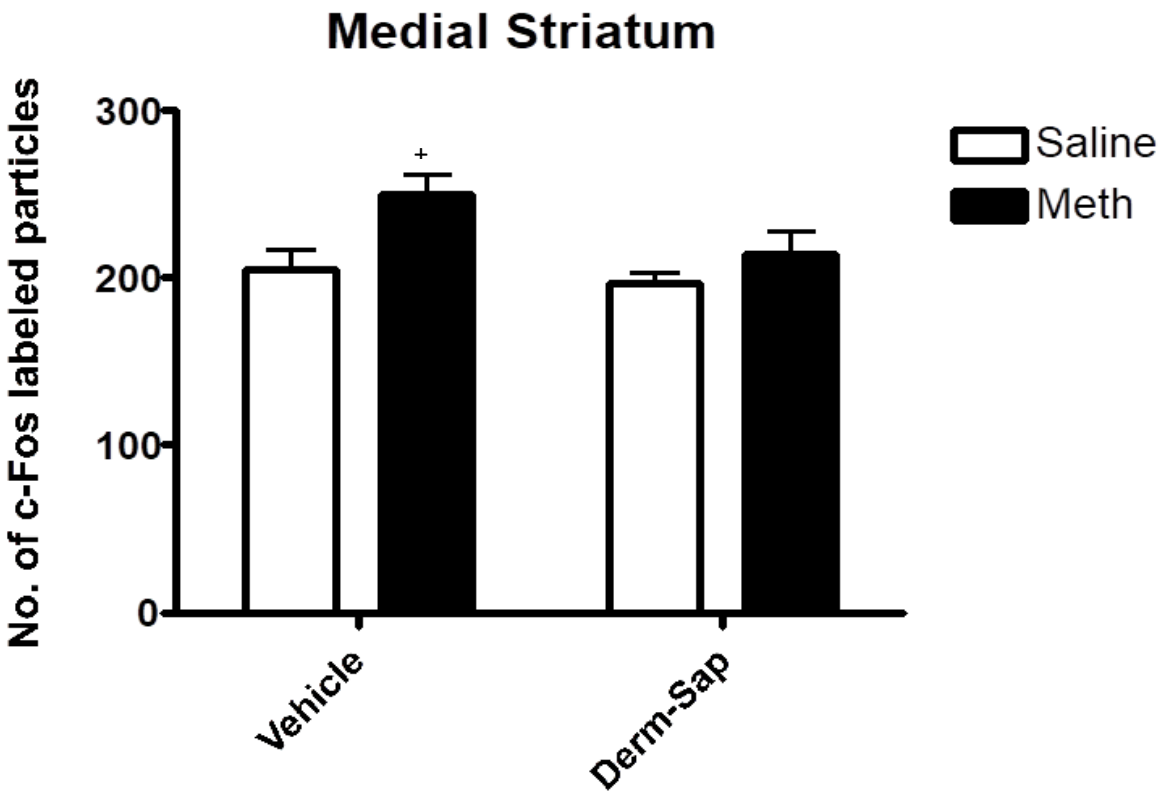

Figure 5: Effects of DERM-SAP pretreatment on c-Fos expression within the dorsomedial striatum. Quantitative results were gathered from a 400 x 400 pixel area in the dorsomedial striatum of all female rats. Analysis shows that there is a significant difference in the number of c-Fos particles between Vehicle/Saline and Vehicle/METH, but not a significant difference in the number of c-Fos particles between DERM-SAP/Saline and DERM-SAP/METH. DERM-SAP prevented METH-induced c-Fos increases in the dorsomedial striatum. +=$p<0.05$ vs. respective saline-treated group.

Effects of DERM-SAP Pretreatment on C-Fos Immunoreactivity in the Sensorimotor Cortex:

After performing a quantitative analysis of the c-Fos particles of the sensorimotor cortex of female rats, a two way ANOVA analysis was run. This analysis of c-Fos immunoreactivity revealed that there was a significant difference between vehicle METH treated animals and vehicle saline treated animals (P=0.0007)(*Figure 6*). Further post-hoc analysis revealed that there was a significant difference in the sensorimotor cortex of female DERM-SAP METH treated animals, and DERM-SAP saline animals (P=0.0495). The analysis of c-Fos levels within the sensorimotor cortex of female rats showed that DERM-SAP pretreatment was unsuccessful in preventing METH-induced c-Fos increases within the sensorimotor cortex.

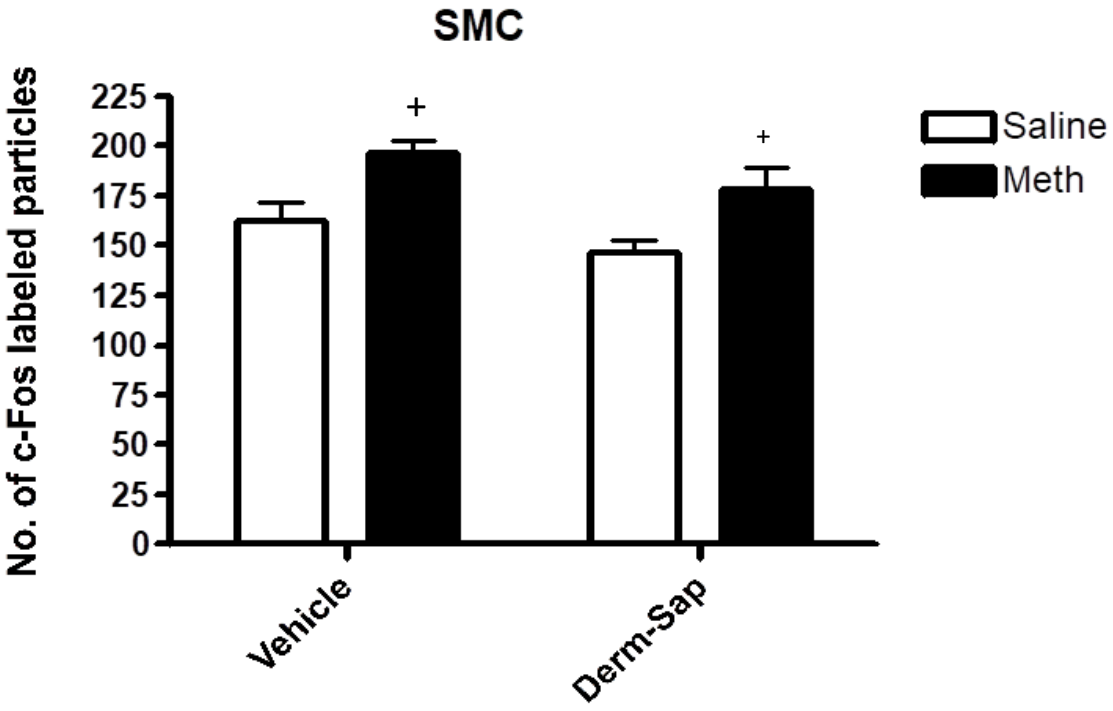

Figure 6: Effects of DERM-SAP pretreatment on c-Fos expression within the sensorimotor cortex. Quantitative results were gathered from a 400 x 400 pixel area in the sensorimotor cortex of all female rats. Analysis shows that there is a significant difference in the number of c-Fos particles between Vehicle/Saline and Vehicle/METH, as well as a significant difference in number of c-Fos particles between DERM-SAP/Saline and DERM-SAP/METH. DERM-SAP did not prevent METH-induced c-Fos increases in the sensorimotor cortex of female rats. +=$p<0.05$ vs. respective saline-treated group

Effects of DERM-SAP Pretreatment on C-Fos immunoreactivity Dorsolateral Striatum:

After performing a quantitative analysis of the c-Fos particles of the dorsolateral striatum of female rats, a two way ANOVA analysis was run. This analysis of c-Fos immunoreactivity revealed that there was a significant difference between vehicle METH treated animals and vehicle saline treated animals (P=0.0257)(*Figure 7*). Further post-hoc analysis revealed that there was no significant difference in the dorsolateral striatum of female DERM-SAP METH treated animals, and DERM-SAP saline animals (P=0.1963). The analysis of c-Fos levels within the dorsolateral striatum of female rats showed that DERM-SAP pretreatment was successful in preventing METH-induced c-Fos increases within the prefrontal cortex.

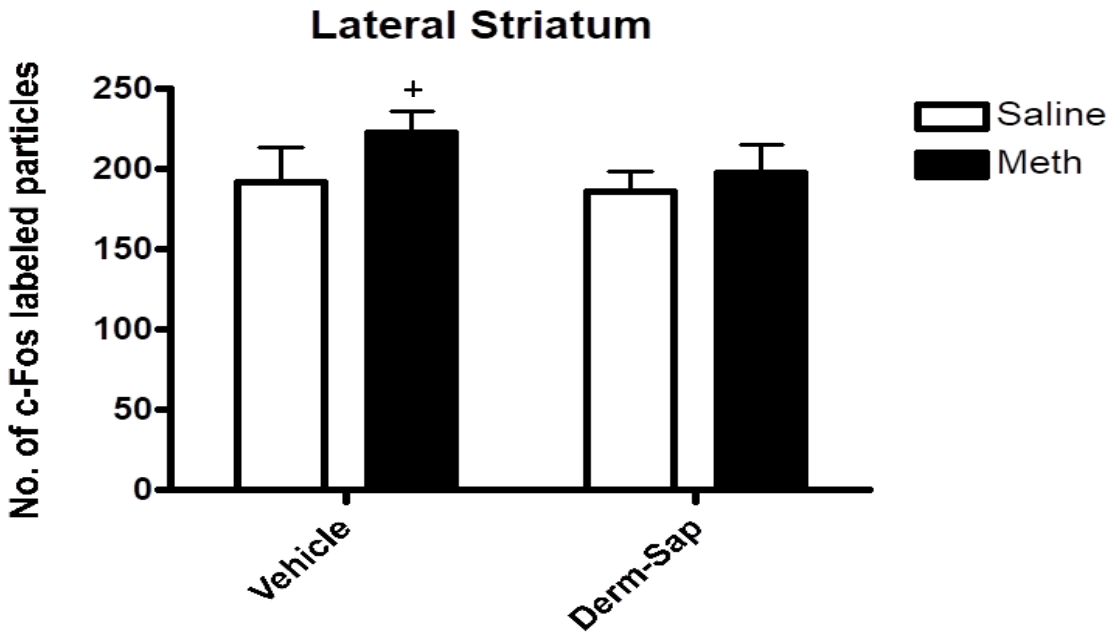

Figure 7: Effects of DERM-SAP pretreatment on c-Fos expression within the dorsolateral striatum. Quantitative results were gathered from a 400 x 400 pixel area in the dorsolateral striatum of all female rats. Analysis shows that there is a significant difference in the number of c-Fos particles between Vehicle/Saline and Vehicle/METH, but not a significant difference in the number of c-Fos particles between DERM-SAP/Saline and DERM-SAP/METH. DERM-SAP prevented METH-induced c-Fos increases in the dorsolateral striatum. +=$p<0.05$ vs. respective saline-treated group

Estrous Cycle Results:

Analysis of the estrous cycle data for the Vehicle METH treated female group showed that each rat was able to finish at least one complete cycle during experimentation. Although some rats were acyclic this data allows for the possible correlation of hormone surges for each rat within their respective group. The majority of females in this group went through a second day of diestrus on day 2 of conditioning in which they received METH. This majority also experienced a second day of diestrus on the final preference testing day which they received no drug (*Figure 8*). The latter portion of the diestrus phase is associated with an increase and peak of the hormone estradiol.

Vehicle/METH	R4-2/18/17	R7-2/18/17	R3-3/10/17	R1-7/29/17	R2-7/29/17	R4-7/29/17
Bias Testing	Proestrus	Proestrus	Diestrus	Metestrus	Metestrus	Metestrus
Day 1	Estrus	Estrus	Proestrus	Diestrus	Diestrus	Diestrus
Day 2	Metestrus	Metestrus	Estrus	Diestrus	Diestrus	Diestrus
Day 3	Metestrus	Metestrus	Metestrus	Diestrus	Diestrus	Diestrus
Day 4	Diestrus	Diestrus	Metestrus	Proestrus	Proestrus	Proestrus
Day 5	Diestrus	Diestrus	Diestrus	Estrus	Estrus	Estrus
Day 6	Diestrus	Diestrus	Diestrus	Metestrus	Metestrus	Metestrus
Day 7	Diestrus	Diestrus	Diestrus	Metestrus	Metestrus	Metestrus
Day 8	Proestrus	Proestrus	Diestrus	Diestrus	Diestrus	Diestrus
Preference test	Estrus	Estrus	Diestrus	Diestrus	Diestrus	Diestrus

Figure 8: Progressive phases of the estrus cycle for the animals used in the Vehicle METH treated group. Some animals were acyclic but all females completed at least one estrous cycle prior to euthanasia.

DISCUSSION

The initial goal of this study was to investigate the role of the patch compartment neurons in METH-induced reward behavior. We also aimed to determine if the ablation of mu opioid receptor-containing neurons of the patch compartment would alter METH-mediated CPP and to attain information on the involvement of the dorsolateral striatum in drug seeking and reward behaviors. CPP was used to accurately determine if the patch compartment of the DLS contributes to reward and drug seeking behavior. It was found that METH-mediated CPP had a reduced effectiveness in female rats which were pretreated with DERM-SAP, while METH-mediated CPP was increased in male rats with DERM-SAP lesions. These results allow us to conclude that patch compartment neurons are necessary for METH-induced reward behaviors in females. We can also conclude that the mu opioid receptors of the patch compartment were essential for reward association and drug seeking behavior in these female rats. Our investigation of the goal directed and habitual behavior basal ganglia circuits within our female subject group yielded us further knowledge of the involvement of the patch compartment amongst these pathways.

While previous studies have provided evidence that the dorsolateral striatum is necessary for habit formation in sucrose self-administration studies(Yin et al., 2004), no studies have implicated a direct involvement of the dorsolateral striatum in METH-mediated reward and drug seeking, which are inflexible behaviors similar to habitual behavior. Varying c-Fos levels amongst differing sections of the basal ganglia circuits allows for the delegation of which circuits are more active during experimentation.

C-Fos data in METH treated animals demonstrated an increase in both the goal directed basal ganglia circuit, which involves the prefrontal cortex and dorsomedial

striatum, as well as the habitual behavior basal ganglia circuit, which runs through the sensorimotor cortex and the dorsolateral striatum. These data showed that METH-induced CPP significantly increased c-Fos activity in vehicle METH treated female rats in both basal ganglia circuits. Knowing this information, we can conclude that METH-mediated reward and drug seeking behavior not only involves the habitual aspects of the DLS, but also involves goal directed priorities of the DMS. These data was interesting given the prevailing theory of habitual drug use by Everitt and Robbins, which states that the enhancement of stimulant induced dopaminergic (DA) activity in the DLS increases the degree in which drug seeking and drug taking behaviors are under the control of stimulus response association (Everitt & Robbins, 2005; Schneck & Vezina, 2012). Our data has shown that the DMS also has some involvement in reward and drug seeking aspects of METH-mediated drug use.

It is possible that the patch circuits which traverse the striatum may be the key link in connecting limbic based association and the basal ganglia circuit. It is well known that neurons of the patch almost exclusively project to the dopaminergic neurons of the substantia nigra pars compacta (Gerfen, 1984; Jimenez-Castellanos & Graybiel, 1987). The patch compartment is critically positioned to gain from mesencephalic dopaminergic neurons as well as affect dopaminergic neurotransmission to regulate basal ganglia output (Canales, 2005). Knowing that the dorsomedial striatum interacts dynamically with projections from the prefrontal cortex sub regions to mediate flexible behaviors; It could be proposed that the limbic interactions of the prefrontal cortex could also be responsible for mediating reward and drug seeking behaviors of the goal-directed basal ganglia loop (Ragozzino, 2007). The prefrontal cortex has been previously believed to have some

involvement in regulation of limbic based reward regions and higher order thinking (Goldstein & Volkow, 2011). With the increase in activation in both the goal directed and habitual behavior basal ganglia loops in METH-mediated drug seeking and reward, it is clear that limbic connections of the patch compartment throughout the striatum work to mediate and regulate these behaviors.

Ablation of the patch compartment in female rats reduced METH-induced increases in c-Fos levels in the PFC, DLS, DMS, but not within the SMC. A clear increase in the habitual behavior loop as well as the goal directed loop following METH-mediated CPP, shows that the increase in activity in both these basal ganglia loops was vital in the association of drug seeking and reward behavior. While we expected to see a preferential increase in only the habitual behavior basal ganglia loop, it was insightful to see also an increase in the goal directed pathway after METH-mediated CPP. Our hypothesis that DERM-SAP pretreatment would cause an aversion for METH in our rats was correct, and the reduction of METH-induced increases in both basal ganglia loops after DERM-SAP pretreatment has provided us with a detailed starting point for specific future studies. DERM-SAP METH treated rats showed little to no reward and drug seeking behavior. With the SMC projecting primarily to the matrix compartment, it is possible that ablation of the mu opioid receptor rich patch had less of an effect on this compartment (Canales & Graybiel, 2000). It is also possible that loss of the patch compartment could cause an increase in the input of matrix neurons. We know the SMC is primarily connected to the matrix, firing of the striatal matrix neurons works to release tonic inhibition of the thalamus. Thus, activation of matrix neurons may be necessary for the regulation of normal sensorimotor patterns and behavioral adaptations.

The clear differences within our female and male data sets may be explainable by the differing hormones of each sex as well as some anatomical brain differences. It has been proven that ovarian hormones can mediate psychostimulant drug effects (Walker et al., 2012). Previous studies have also indicated that female rats have an increased number of midbrain dopamine neurons as well as greater dopamine uptake and release rates (Walker et al., 2012). It has been proposed, but not fully proven, that estradiol and progesterone are both involved in contributing to sex differences in psychostimulant use. During the estrous cycle of rats, estradiol peaks at the end of the last day of diestrus just prior to proestrus, while progesterone has been seen to peak between proestrus and estrus (Goldman et al., 2007). Estradiol has been implicated in amphetamine stimulated dopamine release and in the expression of dopamine transporters and receptors (J. B. Becker, 1999; Jill B. Becker & Hu, 2008). Estrogen works to enhance neurochemical psychostimulant responses in female rats, meaning that female rats have a greater sensitization for psychostimulants than male rats due to gonadal hormones (J. B. Becker, 1999). A previous study has also proposed that estrogen is able to induce neuronal excitability within the striatal neurons and DA terminals, which in turn will work through inhibition of GABAergic receptors stimulating an increased DA release (J. B. Becker, 1999). These astute differences in the neuronal functionality of male and female rats may begin to explain the differing behavioral data we received between the two sexes. According to what we know about hormonal correlation with estrous cycles, our Vehicle METH treated female rats went through an estradiol surge on Day 2 of conditioning in which they received METH. Another possible surge of estradiol for this group of rats was on the day of the preference test. This surge in estradiol could possibly affect the striatal

dopamine release in turn increasing METH-induced excitability of the patch compartment, making it easier for female rats to illicit drug seeking and reward behaviors. Increased DA neuron excitability within the striatum of female rats may be responsible for the METH-induced c-Fos increase seen in the DMS, opposed to just the DLS. Given that DERM-SAP was able to cause a preference aversion in the METH treated female group, as we hypothesized, but not in the male METH treated group. It may be plausible that ablation of mu opioid receptors of the patch compartment in female rats, caused a greater disruption of the dopamine flow of the basal ganglia circuits which are necessary for METH-induced reward association and drug seeking behaviors. With no clear male hormone association with increased dopamine striatal excitability or differences in GABAergic input, male rats are able to associate reward and participate in drug seeking behaviors with less patch compartment activity and fewer mu opioid receptors than females.

In conclusion our lab has been the first to associate the role of the patch compartment with both goal directed and habitual behavior in METH-mediated reward. The chemical ablation of mu opioid receptor containing neurons of the patch compartment alter METH- mediated CPP, causing a reduction in reward and drug seeking behaviors. These data speak to the rewarding and habitual aspects which encompass drug use and perpetuate the cycle of addiction. More research is needed to further investigate the differences in the basal ganglia circuits of addiction in male and female rats.

REFERENCES

Adams, C. D., & Dickinson, A. (1981). Instrumental responding following reinforcer devaluation. *The Quarterly Journal of Experimental Psychology Section B*, *33*(2), 109–121. https://doi.org/10.1080/14640748108400816

Adinoff, B. (2004). Neurobiologic Processes in Drug Reward and Addiction. *Harvard Review of Psychiatry*, *12*(6), 305–320. https://doi.org/10.1080/10673220490910844

Balleine, B. W., & Dickinson, A. (1998). Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. *Neuropharmacology*, *37*(4–5), 407–419.

Barker, J. M., Taylor, J. R., & Chandler, L. J. (2014). A unifying model of the role of the infralimbic cortex in extinction and habits. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *21*(9), 441–448. https://doi.org/10.1101/lm.035501.114

Becker, A., Grecksch, G., Kraus, J., Loh, H. H., Schroeder, H., & Höllt, V. (2002). Rewarding effects of ethanol and cocaine in μ opioid receptor-deficient mice. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *365*(4), 296–302. https://doi.org/10.1007/s00210-002-0533-2

Becker, J. B. (1999). Gender differences in dopaminergic function in striatum and nucleus accumbens. *Pharmacology, Biochemistry, and Behavior*, *64*(4), 803–812.

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

Brown, L. L., Feldman, S. M., Smith, D. M., Cavanaugh, J. R., Ackermann, R. F., & Graybiel, A. M. (2002). Differential metabolic activity in the striosome and matrix compartments of the rat striatum during natural behaviors. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *22*(1), 305–314.

Canales, J. J. (2005). Stimulant-induced adaptations in neostriatal matrix and striosome systems: Transiting from instrumental responding to habitual behavior in drug addiction. *Neurobiology of Learning and Memory*, *83*(2), 93–103. https://doi.org/10.1016/j.nlm.2004.10.006

Canales, J. J., & Graybiel, A. M. (2000). A measure of striatal function predicts motor stereotypy. *Nature Neuroscience*, *3*(4), 9.

Chersi, F., & Pezzulo, G. (2012). Using hippocampal-striatal loops for spatial navigation and goal-directed decision-making. *Cognitive Processing*, *13 Suppl 1*, S125-129. https://doi.org/10.1007/s10339-012-0475-7

Coutureau, E., & Killcross, S. (2003). Inactivation of the infralimbic prefrontal cortex reinstates goal-directed responding in overtrained rats. *Behavioural Brain Research*, *146*(1–2), 167–174.

Dickinson, A. (1985). Actions and habits: the development of behavioural autonomy. *Phil. Trans. R. Soc. Lond. B*, *308*(1135), 67–78. https://doi.org/10.1098/rstb.1985.0010

Everitt, B. J., & Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nature Neuroscience*, *8*(11), 1481–1489. https://doi.org/10.1038/nn1579

Garrett, B. E., & Holtzman, S. G. (1996). Comparison of the effects of prototypical behavioral stimulants on locomotor activity and rotational behavior in rats. *Pharmacology, Biochemistry, and Behavior*, *54*(2), 469–477.

Gerfen, C. R. (1984). The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. *Nature*, *311*(5985), 461.

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, *80*(2), 84–97. https://doi.org/10.1002/bdrb.20106

Goldstein, R. Z., & Volkow, N. D. (2011). Dysfunction of the prefrontal cortex in addiction: neuroimaging findings and clinical implications. *Nature Reviews. Neuroscience*, *12*(11), 652–669. https://doi.org/10.1038/nrn3119

Graybiel, A. M. (1990). Neurotransmitters and neuromodulators in the basal ganglia. *Trends in Neurosciences*, *13*(7), 244–254.

Guix, T., Hurd, Y. L., & Ungerstedt, U. (1992). Amphetamine enhances extracellular concentrations of dopamine and acetylcholine in dorsolateral striatum and nucleus accumbens of freely moving rats. *Neuroscience Letters*, *138*(1), 137–140.

Haber, S. N. (2016). Corticostriatal circuitry. *Dialogues in Clinical Neuroscience*, *18*(1), 7–21.

Harris, M. A., & Kesel, M. L. (1990). An improved method for accurately timed mating in rats. *Laboratory Animal Science*, *40*(4), 424–425.

Horner, K. A., Logan, M. C., Fisher, T. J., & Logue, J. B. (2017). Blockade of patch-based μ opioid receptors in the striatum attenuates methamphetamine-induced conditioned place preference and reduces activation of the patch compartment. *European Journal of Pharmacology*, *796*, 207–214. https://doi.org/10.1016/j.ejphar.2017.01.001

Jimenez-Castellanos, J., & Graybiel, A. m. (1987). Subdivisions of the dopamine-containing A8-A9-A10 complex identified by their differential mesostriatal innervation of striosomes and extrastriosomal matrix. *Neuroscience*, *23*, 223–242. https://doi.org/10.1016/0306-4522(87)90285-5

Kravitz, A. V., & Kreitzer, A. C. (2012). Striatal Mechanisms Underlying Movement, Reinforcement, and Punishment. *Physiology (Bethesda, Md.)*, *27*(3). https://doi.org/10.1152/physiol.00004.2012

Lohmiller, J. J., & Swing, S. P. (2006). Chapter 6 - Reproduction and Breeding. In M. A. Suckow, S. H. Weisbroth, & C. L. Franklin (Eds.), *The Laboratory Rat (Second Edition)* (pp. 147–164). Burlington: Academic Press. https://doi.org/10.1016/B978-012074903-4/50009-1

McGeorge, A. J., & Faull, R. L. (1989). The organization of the projection from the cerebral cortex to the striatum in the rat. *Neuroscience*, *29*(3), 503–537.

NIDA. (n.d.). Drugs, Brains, and Behavior: The Science of Addiction. Retrieved from https://www.drugabuse.gov/publications/drugs-brains-behavior-science-addiction

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

Pan, W. X., Mao, T., & Dudman, J. T. (2010). Inputs to the dorsal striatum of the mouse reflect the parallel circuit architecture of the forebrain. *Frontiers in Neuroanatomy*, *4*, 147. https://doi.org/10.3389/fnana.2010.00147

Parent, A., Cicchetti, F., & Beach, T. G. (1995). Calretinin-immunoreactive neurons in the human striatum. *Brain Research*, *674*(2), 347–351.

Park, S. W., Shen, X., Tien, L.-T., Roman, R., & Ma, T. (2011). Methamphetamine-induced changes in the striatal dopamine pathway in μ-opioid receptor knockout mice. *Journal of Biomedical Science*, *18*(1), 83. https://doi.org/10.1186/1423-0127-18-83

Ragozzino, M. E. (2007). The Contribution of the Medial Prefrontal Cortex, Orbitofrontal Cortex, and Dorsomedial Striatum to Behavioral Flexibility. *Annals of the New York Academy of Sciences*, *1121*(1), 355–375. https://doi.org/10.1196/annals.1401.013

Schneck, N., & Vezina, P. (2012). Enhanced Dorsolateral Striatal Activity in Drug Use: The Role of Outcome in Stimulus-Response Associations. *Behavioural Brain Research*, *235*(2), 136–142. https://doi.org/10.1016/j.bbr.2012.07.042

Walker, Q. D., Johnson, M. L., Van Swearingen, A. E. D., Arrant, A. E., Caster, J. M., & Kuhn, C. M. (2012). Individual differences in psychostimulant responses of female rats are associated with ovarian hormones and dopamine neuroanatomy. *Neuropharmacology*, *62*(7), 2267–2277. https://doi.org/10.1016/j.neuropharm.2012.01.029

White, N. M., & Hiroi, N. (1998). Preferential localization of self-stimulation sites in striosomes/patches in the rat striatum. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(11), 6486–6491.

Wise, R. A. (1996). Addictive drugs and brain stimulation reward. *Annual Review of Neuroscience*, *19*, 319–340. https://doi.org/10.1146/annurev.ne.19.030196.001535

Yin, H. H., & Knowlton, B. J. (2006). The role of the basal ganglia in habit formation. *Nature Reviews Neuroscience*, *7*(6), 464–476. https://doi.org/10.1038/nrn1919

Yin, H. H., Knowlton, B. J., & Balleine, B. W. (2004). Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. *The European Journal of Neuroscience*, *19*(1), 181–189.

Zapata, A., Minney, V. L., & Shippenberg, T. S. (2010). Shift from Goal-Directed to Habitual Cocaine Seeking after Prolonged Experience in Rats. *Journal of Neuroscience*, *30*(46), 15457–15463. https://doi.org/10.1523/JNEUROSCI.4072-10.2010