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Anxiety-Like Behavior in Adolescent Rats Following Maternal Separation and Chronic Cocaine Exposure

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Abstract

Cocaine is one of the most widely used illicit substances in the world and has an addiction rate comparable to opioids. Early Life Stress (ELS) has been shown to have a profound influence on the development of an individual, showing strong correlations to the development of psychiatric disorders and psychostimulant abuse. Adolescents in particular are at a high risk for the abuse of psychostimulants such as cocaine. Previous studies have individually described the correlation between cocaine addiction and anxiety, and the correlation between ELS and cocaine addiction. Rats who have experienced some form of ELS have shown a higher levels of self-administration, but the anxiety resulting from addiction and ELS has not been observed. This study sought to examine the effects of ELS and/or adolescent cocaine exposure on anxiety-like behavior, as measured by the Elevated Plus Maze (EPM) and Open Field tests in rats. Due to complications in maternal separation and behavioral sensitization to cocaine, the relationship these variables have on expressed anxiety-like behavior is unclear.

Introduction

Substance abuse and anxiety comorbidity

With an addiction rate comparable to opioids, cocaine and cocaine-derived drugs prove to be some of the most widely used illicit substances in the world. The SAMHSA 2014 National Survey on Drug Use and Health revealed that approximately 1.5 million people in the US have used cocaine (either in the powder cocaine form or crack cocaine form). Psychostimulants such as cocaine have been shown to greatly affect the reward neural circuitry pathway as well as the brain areas most associated with learning and memory, and thus can develop strong drug-seeking behavior when stimulants are used chronically (Taylor, 2013). Rat models of cocaine addiction have indicated that during a period of abstinence, an individual addicted to cocaine can express increased levels of anxiety, making relapse all the more likely even when in a treatment program (Hage et al., 2012). As well as having high rates of addiction, psychostimulants also tend to follow a pattern of behavioral sensitization, meaning continued substance exposure will elicit greater changes in behavior without an increase in the amount of substance used.

Substance abuse and anxiety disorders are two very commonly comorbid conditions, and have been the subject of intense psychiatric study (Smith and Book, 2008). Smith and Book also suggest in their review of the comorbidity of Substance Use Disorder (SUD) and Anxiety Disorders (ADs), that these two conditions develop and are maintained in tandem. A model of co-development would suggest that SUD can develop due to a pre-existing AD, and that an AD can develop due to the acquisition of substance dependence. Psychostimulants, such as cocaine, tend to follow this comorbid trend quite closely, as their abuse tends to incite a euphoric rush. Once the euphoria comes to an end, and the effects of the psychostimulant have worn off, the

individual can be left with residual symptoms of anxiety. This phenomenon presents a problem to those suffering from any form of substance use disorder, as the increased level of anxiety induced by the lack of exposure to his or her drug of abuse would act as a source of psychological encouragement to continue a pattern of abuse. Acute psychological stress has has also been demonstrated to significantly increase drug-craving behavior (Sinha, Catapano, & O'Malley, 1999). When acute stress increases drug-craving behavior, the stressor begins to feed into the cycle of addiction, which likely induces a cycle of stress and anxiety as well. If an individual were to express a heightened stress response, this issue of addiction could be all the worse. The correlation between anxiety disorders and SUD has been documented to be significantly positive, which clearly alludes the detrimental cycle that surrounds SUD (Brady et al., 2010). With such a strong correlation between ADs and SUDs, it becomes apparent that these two disorders need to be studied in tandem as well as suggesting that their influences on each other need to be defined.

Early Life Stress

Early Life Stress (ELS) has been shown to have profound influence on the development of an individual, showing strong correlations with the development of psychiatric disorders and significantly altered biochemical markers. One of the most prominent biomarkers in the evaluation levels of stress and anxiety is the presence of corticotropin-releasing factors (CRF). Corticotropin-releasing factor is the primary regulators of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Smith and Vale 2006, Syed and Nemeroff, 2017). CRF has been demonstrated as having strong influences on the psychophysiological responses to stress, going as far as to change the eating and sleep patterns of rats when CRF was injected into their central nervous

system (Syed and Nemeroff, 2017). When The HPA axis consistently releases CRF to the point of HPA axis dysregulation, as is consistent with the current understanding of chronic stress, brain development can be significantly impaired. Chronic stress during early stages of development have also been demonstrated to significantly impact the development of the HPA axis. Individuals with this altered HPA axis have been shown to a significantly altered response to stressors compared to individuals who never experienced early life stress (van Bodegom, 2017). The resulting impairment can include the myelination of various different neurological structures (Brietzke, 2012). Any impairment in neurological development has the potential to have a much greater impact when the impairment occurs early in life, such as in childhood. Magnetic resonance images comparing the brains of school-aged children to those of adults have revealed a significant increase in myelination during childhood and adolescence (Jernigan et al., 2011), and if this period of increased myelination were to be interrupted by a disruption of the HPA axis, the resulting effects have the potential to be both long lasting and detrimental. The biological impacts of ELS do not only impact the brain however, as it the experienced trauma or neglect has been shown to have fairly extensive impacts on the immune system (Elwenspoek, 2017). ELS has been shown to have a lasting impact on development, both at a hormonal and neuroanatomical level.

Early life stress is well documented as being a strong predictor for the development of psychiatric disorders, particularly anxiety and depressive disorders (Nugent et al., 2010, Enoch, 2010). Bradley et al. (2008) demonstrated that individuals who have experienced childhood trauma scored significantly higher on Beck's Depressive Index (BDI), and managed to use polymorphisms in the corticotropin-releasing hormone gene as predictors for higher BDI scores.

Given the intertwined development of SUD with ADs, ELS has a clear impact on the development of substance addiction later on in life. In a study conducted by al'Absi, Nakajima, and Lemieux, individuals who have experienced ELS were found to have more severe withdrawal symptoms in the form of reporting more pain after being exposed to a pain stimulus (2018). These findings suggest that the heightened level of anxiety of individuals that have experienced high levels of ELS in their lifetime have a strong influence on their anxiety. The relationship between drug addiction and anxiety can be defined as a distress-addiction cycle, meaning that the presence of a stressor can increase drug seeking behavior, thereby furthering drug exposure and addiction. Within the brain, this system is thought to be established by the interactions of the HPA axis and the mesolimbic system (Sinha, 2008). Operating under a distress-addiction cycle model of addiction provides a sensible explanation as to why ELS individuals are more susceptible to drug addiction. Their dysfunctional HPA axis established by a previous childhood trauma or neglect would result in increased CRF levels that could be alleviated by the use of a drug such as opioids or psychostimulants. Once the effects of the drug wear off and the individual is presented with an additional stressor, whether it be physical or psychosocial, the individual would likely experience comparable or higher CRF levels, pushing them to alleviate their symptoms again with the use of the same drug.

Risk of Cocaine Addiction and ELS

Neighborhood lower socioeconomic status correlates strongly with drug addiction rates in a population, suggesting that those with fewer resources and poor living conditions lead to a greater chance of cocaine addiction as well as physiological dysregulation that lead to an increase in allostatic load (Williams, 2007; Schroeder et al., 2001; Ribeiro et al., 2018). The

stress caused by growing up in these conditions can be simulated in rats by the various Early Life Stress models, one of which is Maternal Separation (MS) (Molet et al., 2014; Lewis et al. 2016). In this model of stress, rat pups are separated from their mother for a variable length of time, typically once a day for the first two weeks of life. Adult rats that have been subject to this model have shown an increase of anxiety-like behavior when placed in conditions such as the elevated plus maze (Wang et al., 2017). This model provides a seemingly stable level of anxiety into adulthood for rats that are subject to 180 minutes of separation for the first two weeks after birth. Murthy and Gould (2018) suggest that the MS model has a number of issues with uncontrollable variability, including the ability of the dam from each group to provide compensating attention to the pups after each separation. While this variation in care as a response to separation does have face-validity for humans as human mothers raising children in the same conditions can provide varying levels of care, it can make results from the rat model of MS more difficult to interpret.

Addiction in Adolescence

Adolescence presents a period of development during which an individual is considered to be at a much greater risk for being exposed to addictive substances and developing an addiction to these substances. Wong et al.'s study examining cocaine self-administration in adolescent rats revealed that adolescence does present a developmental stage in which cocaine addiction is acquired more easily and faster than during adulthood. Dopamine pathways in adolescent rats also demonstrated a higher level of activity, further implicating the high risk of adolescent cocaine use when compared to use during adulthood (Wong et al., 2013). As well as having a high risk of addiction, approximately 30% of adolescents suffer from one or more anxiety disorder (National Institute of Mental Health). This extremely high prevalence of anxiety

disorders and the strong comorbidity of SUD with ADs illuminate why adolescence is a reasonable target for anxiety and addiction studies. This becomes even more apparent when ELS is taken into consideration as a common cause for the development of a variety of anxiety disorders. There is a strong likelihood that the development of some form of anxiety disorder as the result of a childhood experiencing ELS could result in the development of some form of SUD. With the distress-addiction cycle in mind, it is possible that a heightened level of anxiety from an AD caused by ELS could enhance drug-seeking behavior due to a dysregulation in the HPA axis and the cascade of interactions that follow in the mesolimbic system.

Cocaine Pharmacology

Substances of abuse such as cocaine and morphine often result in a pattern of behavior known as behavioral sensitization. In rats, sensitization essentially has the opposite effect of tolerance and can be observed as an increase in locomotor activity with each subsequent administration of cocaine. This behavioral trend is considered to be one aspect of psychostimulant addiction development and has been shown to have lasting effects in rats long after the most recent exposure (Marin, 2008, Schoffelmeer, 2002). Psychostimulants such as cocaine and amphetamine are believed to mediate the increase of psychomotor activity through the intermittent increase of dopamine in the synaptic space in the medial prefrontal cortex (mPFC). This dopamine pathway begins at the Ventral Tegmental Area (VTA), which has been shown to be the area of the brain that interacts with psychostimulants. Activity in the mesolimbic pathway has demonstrated as having an influence on expressed anxiety. Some studies have demonstrated that stress appears to be induce the release of dopamine metabolites in the nucleus accumbens and the mPFC (Sorg and Kaivas, 1993). Habituation of psychostimulants is believed

to be mediated by the dorsal striatum. In the beginning uses of a drug such as cocaine, the VTA would send send an excess of DA to the NAc shell, and then later to the NAc core. At this point in habitual drug use, more DA is sent to the DS, and glutamatergic transmission within the DS has been implicated to be important in drug-induced adaptations (Taylor, 2013). While the nigrostriatal pathway also contain dopaminergic neurons, it is not believed to be involved in addiction acquisition, but rather in the locomotor expression of behavioral sensitization. This was demonstrated in a study conducted by Beeler et al., using *Pitx3*-deficient mice, meaning the mice had no functioning nigrostriatal pathway (2008). The proposition that repeated exposure to stress induces increased dopamine metabolism provides further understanding to the current understanding of psychostimulant addiction and the way in which it interacts with ELS. However, more recent investigations of dopamine inhibitors, such as Monoamine Oxidase A (MAO-A), have found that acute psychosocial stressors result in a decrease in MAO-A binding in human brain (Soliman et al., 2012). Conflicting implications of MAO-A production during stress complicates the role of dopamine in the mesocortical pathway as it relates to stress, and thereby mystifies the direct connection between stress and psychostimulant addiction.

ELS in Rats

Animal models of ELS and addiction have furthered our understanding of the relationship between the two behavioral factors. Rats with that have experienced maternal separation for 180 minutes each day of their first two weeks of life managed to acquire cocaine-self administration at a low test dose whereas groups of rats that received “maternal handling” (15 minutes of separation with handling) did not (Moffet et al., 2007). The same study also confirmed that the lower dosage threshold for self administration was not a result of lower drug metabolism by

measuring the levels of liver carboxylesterase, which did not differ between the MS group and the control group. Varghese et al. sought to explore this idea when they developed their own model of ELS by developing a mouse model where CRF was overexpressed, and then measuring their morphine sensitization and withdrawal symptoms when compared to wild type (2015). Although this study may not have the same proportional level of CRF that would be present in rats that experienced ELS similar to the MS model, it does prevent a lot of the unpredictability involved with the variations in maternal rearing as a response to separation. After overexpressing CRF in mice, Varghese et al. determined that the synthetic ELS mice experienced significantly higher morphine sensitization and withdrawal symptoms. Using a mouse model specifically altered to produce an increase in CRF helps to confirm what was determined in previous studies on humans while also establishing a relationship between the HPA axis and drug-addiction behavior. Research conducted by Hynes et al. revealed that rats who were exposed to a number of different forms of early life adversity immediately after weaning (predator odor, restraint stress, forced swim), were more sensitive to reward cues even without exposure to an addictive substance (2017). The determination that rats were more sensitive to a non-addictive reward cue demonstrates the powerful baseline influence early life adversity can have on an animal. If the non-addictive reward cue were to be replaced with a psychostimulant such as amphetamine or cocaine, which directly impact the reward pathway by blocking dopamine transporter, the increase of reward sensitivity would likely be even more pronounced. The heightened baseline reward sensitivity in rats that have experienced adversity is also useful to understanding the Distress-Addiction cycle, as it provides a glimpse into the incentives behind taking the drug for an ELS individual. Prior to drug exposure, if an ELS individual was already sensitive to rewards,

the euphoric rush of abusing a drug such as cocaine would be even greater than an average person. This experience, in tandem with the already heightened baseline of CRF that makes the highs seem higher and the lows seem lower, would create a self-perpetuating cycle of abuse.

Anxiety-like Behavior Assays in Rats

The elevated plus-maze (EPM) is a commonly used behavioral assessment of anxiety-like behavior in rats. EPM tests allow researchers to observe the anxiolytic or anxiogenic effects of a variety of stressors and pharmacological intervention, while maintaining face validity by taking advantage of rats' natural fear of heights and open spaces (Walf and Frye, 2007). A study assessing the anxiety-like behavior in adolescent rats after MS took advantage of the EPM to measure anxiety-like behavior (Jin et al., 2018). This particular study only recorded two behaviors from the EPM, those being the number of entries in the open arm and the time spent in the open arm. The adolescent rats exposed to maternal separation showed significantly fewer open arm entries and significantly less time in the open arm, indicating a heightened level of anxiety. Demonstrating a heightened level of anxiety using EPM not only demonstrates the usefulness and validity of EPM as a behavioral assessment, but also provides further evidence that MS can produce anxiety during adolescence.

Another common method of measuring anxiety-like behavior is the open field test (OF). Although OF has been used by since the 1930s to measure "emotionality" in rodents, the actual interpretation of results and how they can relate to emotional states such as anxiety in rodents is a subject of intense debate (Seibenhener and Wooten, 2015). Rather than taking advantage of two innate fears like in the EPM, OF testing is a measure of locomotor activity, where the total distance traveled is measured in some capacity. In addition to locomotor activity, "zones" can be

artificially constructed (such as the edge and center of the open field) to help interpret anxiety-like behavior. Anxiogenic behavior is typically thought to be an increase in the amount of time spend in “edge” zones. The reason for this follows the natural fear of open areas that is also used in the EPM. Jin et al. also used the OF in their analysis of anxiety-like behavior in adolescent rats. The adolescent rats raised with MS conditions were found to cross significantly more of the lines that divided the open field, while simultaneously making fewer entrances into the “central” zone (Jin et al., 2018). This provides a clear demonstration of the difficulties in interpreting locomotor activity as it relates to anxiety. While the MS rats crossed significantly showed significantly more locomotor activity, they also demonstrated a reluctance to enter the central zone of the open field test. Although it was not explicitly stated in Jin et al.’s results, it is most likely that the majority of locomotor activity was expressed outside of the central zone, which would follow the logic of more anxious rats being less likely to overcome the fear of open spaces.

The purpose of this study is to examine how the the relationship between MS and cocaine addiction affect expressed levels of anxiety in adolescent rats. While ELS and cocaine addiction are two well-documented fields of research, their combinatory influence on expressed levels of anxiety has yet to be thoroughly explored. This presents an issue when attempting to assess treatment strategies for an individual who has both experienced ELS and developed an addiction to cocaine. It is possible that the combination of both of these psychosocial stressors could have a cumulative effect and result in a much higher risk of relapse. Without a proper understanding of how the combination of these two stressors interact, individuals attempting to recover from a cocaine addiction may not receive the most appropriate form of treatment to increase their

likelihood of success. In order to quantify anxiety like behavior in this study, the elevated plus maze and open field tests will be used at various stages of the rats' cocaine or saline treatment. The behavioral trends elucidated by this study have the potential to lead to research surrounding the specific biomarkers that contribute to the increasingly anxious behavior with the addition of each additional stressor.

Methods

Research Design

This experiment is a true experimental design. The two independent variables investigated in this study are exposure to Maternal Separation and exposure to cocaine. Maternal separation has two levels (separated and not separated), and cocaine exposure had three levels (0 mg/kg, 10 mg/kg, and 20 mg/kg). The dependent variables for this study based on EPM testing consisted of open arm entries, open arm time (seconds), closed arm entries, protected head dips, unprotected head dips, end-of-arm head dips, and stretch attend. The dependent variables based on Open Field testing consisted of number of squares crossed, and time spent at the edge (seconds).

Subjects

With the use of the Maternal Separation model of Early Life Stress, it was necessary to use 66 Sprague-Dawley rats born from 6 different dams under the supervision of Connecticut College's Animal Care Facility. Newborn pups were raised by their respective dam until they became old enough to be weaned. All animals had ad libitum access to food and water and were housed under standard conditions on a 12 hour on 12 hour off light-dark cycle. One rat died due to a complication during a cocaine injection.

Materials

The elevated plus maze is an apparatus used to analyze anxiogenic compounds as well as for anxiety research in general. The EPM consists of a "plus" shaped structure which includes two closed arms surrounded by walls and two open arms which have no arms around them. The arms are elevated 50 cm above the floor. This model is used due to research that shows rats' aversion to open spaces, and a tendency to remain in the closed arms, as this is a safer space for the animals (Roy et. al, 2009).

The open field is another apparatus used to analyze anxiety-like behavior in mice. The open field apparatus used in this experiment was 2 meters x 2 meters surrounded by a 50 cm wall, and segmented into 20 cm squares with a 5 cm "edge area" extending from each wall. The same aversion of open spaces that applies to the elevated plus maze applies to the open field test, with the "edge area" taking the place of the closed arms as the safer space for the animal to stay.

Cocaine hydrochloride (Sigma-Aldrich) was administered to rats via intraperitoneal injections. The cocaine was dissolved in sterile saline at a concentration of 10 mg/mL or a 20 mg/mL. Rats in the non-cocaine groups were administered an equivalent volume of sterile saline.

Following testing, each rat was euthanized via carbon dioxide exposure as specified by the approved ACUP procedure.

Procedure

Timed-pregnant dams were received at approximately 18 days after conceiving. After each dam was received, their cages were monitored daily for the birth of pups. The day that the pups were born was indicated as Postnatal Day 1 (PND1). Before the pups were born, and throughout the time in which the pups were house with the mother, food and water were

available ad libitum. After the pups were weaned on PND21, they were each housed in groups of three or four with their respective littermates.

Maternal Separation

Starting on PND2, the dams of the three Maternal Separation (MS) groups were housed in a separate cage with access to food and water for a period of 180 minutes, after which the dam was returned to her pups. MS was performed on a daily basis through PND15.

Cocaine injections

Cocaine was dissolved in a saline solution at a concentration of 10 mg/mL and 20 mg/mL. Starting approximately PND28, the rats were weighed and administered their designated dose and appropriate volume of cocaine solution to deliver a 10 mg/kg or a 20 mg/kg dose. All cocaine injections were delivered into the intraperitoneal cavity. In order to mitigate the difference in stressors experienced by each group of rats, the groups that were not designated to receive cocaine were administered an equivalent volume of saline solution. After all injections that were not followed by locomotor activity monitoring, all rats were placed back into their cage with their the same litter mates they had been housed with previously.

Behavioral Sensitization

All rats were subject to locomotor activity testing on their first, seventh and fourteenth day of injection (approximately PND28, PND35, PND42). Locomotor activity was measured by placing injected rats into a standard cage placed inside an activity monitor frame.. Each time the rat moved and interrupted an infrared beam, a counter on the photocell monitor would increase by one. Cages in the photocell monitors were equipped with a minimal amount of bedding in an attempt to not cause any additional stress on the tested rats by putting them in an unfamiliar

setting, while also ensuring that the bedding did not interfere with the photocell monitor's ability to present accurate data. The testing period lasted for 30 minutes, after which the number shown by the photocell monitor was recorded and the rats were returned back to their cages.

Cocaine Extinction

After the fourteenth injection of cocaine, the rats were then put through a 14 day extinction period, during which food and water were still available *ad libitum*.

Open Field Testing

In order to assess expressed anxiety-like behavior, each rat was monitored in the open field at three separate timpoints: the day before cocaine injections started (Baseline), after injections were complete (Post-Inj), the last day of cocaine extinction (Post-Extinction). These OF testing days were planned to take place approximately on PND26, PND43, and PND55. During each trial, rats were allowed to explore the open field freely, and were scored on the amount of time they spent at the edge of the field (in seconds) and the number of squares they crossed in their 5 minute trial.

Elevated Plus Maze Testing

In order to assess their expressed anxiety-like behavior, each rat was monitored on the elevated plus maze on three separate occasions: baseline, after injections were complete (Post-Inj), and after their cocaine extinction had ended (Post-Extinction), for a period of 5 minutes. These EPM testing says were planned to take place approximately on PND27, PND44, and PND56. During each trial, researchers recorded each rat's movements on the maze using LifeCam. LifeCam allowed researchers to record open arm duration, open arm entry, closed arm entry, protected head dips, unprotected head dips, and stretch attend posture. Open arm duration

was defined as the period of time during which all four of the rat's paws were in the open arms. Open and closed arm entry were noted when all four paws were placed in the open or closed arms, respectively. A protected head dip was noted as all four paws were in the closed arm or the center of the maze and their head dipped downwards. An unprotected head dip was defined as having all four paws on the open arm and the head dipped downwards. An end-of-arm head dip was when a rat dipped its head at the end of the open arms. Stretch attend posture was marked when the tested rats would keep their hind paws in one place while slowly moving forward with their front paws, resulting in their body "stretching".

Data Analysis

Once all of the behavioral testing was complete, differences in behavior were analyzed using a two-way ANOVA, followed by a Tukey's multiple comparison test to determine the interaction between all of the behaviors measured in the EPM and OF testing. The pattern of locomotor activity was also analyzed in order to determine if rats exposed to cocaine became behaviorally sensitized during their injection period.

Ethical Issues

Animal subjects were required for this experiment and were approved for use in this study by the Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Results

1. Locomotor Activity

None of the groups exposed to cocaine (Coc10/MS, Coc10/No MS, Coc20/MS, Coc20/No MS) expressed any significant increase in locomotor activity from the second

injection to the third injection. Both of the saline groups saw no significant change in locomotor activity (See Fig. 2).

2. Elevated Plus Maze and Open Field

2.1 First Cohort

Two-way ANOVA analysis (timepoint x group) revealed a significant main effect for the number of open arm entries ($F_{6, 126} = 5.426$, $p = <0.0001$) as well as a significant difference in both the time point ($F_{2, 126} = 23.85$, $p<0.0001$) and group variables ($F_{3, 126} = 15.68$, $p<0.0001$). Tukey's multiple variable comparisons testing revealed a no significant difference in open arm entries for the Coc10/MS group when Baseline, Post-Inj, and Post-Extinction timepoints were compared. Sal/MS showed a significant decrease in the open arm entries when Baseline is compared to the Post-Inj and Post-Extinction time points (Baseline: 6.86 ± 2.73 vs. Post-inj: 2.43 ± 1.62 vs. Post-Extinct: 2.857 ± 1.86, $p<0.0001$). The Coc10/No MS group showed a significant decrease in open arm entries between the post-injection measurement and the post-extinction measurement, as well as the baseline compared to the post-extinction measurement (Baseline: 2.67 ± 1.11 vs. Post-inj: 2.13 ± 1.06 vs. Post-extinct: 0.67 ± 0.62, $p<0.005$ and $p<0.01$ respectively). The Sal/No MS group showed no significant differences in the number of open arm entries made while on the EPM.

Between groups Tukey post-hoc comparisons revealed that the Sal/MS group made significantly more open arm entries compared to the Coc10/MS group at baseline (Sal/MS: 6.86 ± 2.73 vs. Coc10/MS: 1.50 ± 2.73, $p<0.0001$). The Coc10/MS group and the Coc10/No MS groups had no significant difference in the number of open arm entries made during the baseline measurement. The Coc10/MS and the Sal/No MS group also had no significant difference of

open arm entries during the baseline test. The Sal/MS group was revealed to have made significantly more open arm entries than Coc10/No MS (Sal/MS: 6.86 ± 2.73 vs. Coc10/No MS: 2.67 ± 1.12, $p<0.0001$) When Sal/MS was compared to Sal/No MS, the Sal/MS group was revealed to have made significantly more open arm entries (Sal/MS: 6.86 ± 2.73 vs. Sal/No MS: 3.17 ± 1.03, $p<0.0001$). The Coc10/No MS and Sal/No MS groups had no significant difference in open arm entries ar baseline. There was no significant differences between any groups at the post-injection stage of testing. After the post-extinction stage of testing the Coc10/MS and Sal/MS groups, as well as the Coc10/MS and COC10/No MS has no significant difference in the number of open arm entries made during the five minutes on the EPM. Coc10/MS and the Sal/No MS groups had no significant difference in the number of open arm entries made in the EPM after their 14 day extinction period. The Sal/MS group did however make significantly more entries compared to the Coc10/No MS group (Sal/MS: 2.86 ± 1.86 vs. Coc/No MS: 0.67 ± 0.62, $p<0.005$), and the Coc10/No MS group made significantly fewer entries than the Sal/No MS group (Coc10/No MS: 0.67 ± 0.62 vs. Sal/No MS: 3.17 ± 1.03, $p<0.0001$). The Sal/MS groups and Sal/No MS groups has no significant difference in the number of open arm entries made while on the EPM (See Fig. 3).

Two-way ANOVA analysis (timepoint x group) revealed a significant main effect for the number of closed arm entries ($F_{6, 126} = 3.088$, $p = <0.01$) and a significant difference in both the time point ($F_{2, 126} = 24.72$, $p<0.0001$) and group variables ($F_{3, 126} = 12.48$, $p<0.0001$). Tukey's multiple comparisons testing within each of the groups revealed that the Coc10/MS group had significantly fewer closed arm entries at baseline compared to the post-extinction round of testing (Baseline: 2.92 ± 1.56 vs. Post-Extinct: 1.50 ± 0.52, $p<0.0001$). There were no significant

difference in the number of closed arm entries made between the baseline and post-injection tests for the Coc10/MS group, nor was there a difference when the post-injection trial was compared to the post-extinction test. The Sal/MS group had a significant decrease in the number of closed arm entries after the baseline trail when compared to both the post-injection and post-extinction trials (Baseline: 5.86 ± 1.95 vs. Post-inj: 2.86 ± 1.77 vs. Post-Extinct: 2.57 ± 1.27, both comparisons $p<0.0001$). The Coc10/No MS group showed significantly fewer closed arm entries after extinction when compared to the baseline and post-injection trials (Baseline: 3.13 ± 1.06 vs. Post-inj: 2.27 ± 0.96 vs. Post-Extinct: 1.07 ± 0.46, $p<0.0001$ and $p<0.05$ respectively). There was no significant difference in closed arm entries for the Coc10/No MS group when the baseline trial is compared to the post-injection trial. The Sal/No MS group only showed only a significant decrease in the number of closed arm entries from the baseline test to the post-injection test (Baseline: 4 ± 1.06 vs. Post-inj: 2.67 ± 1.67, $p<0.05$). Neither the closed arm entries observed during the baseline nor the post-injection EPM trials were significantly different than the closed arm entries observed in the post-extinction trial.

Between group comparisons of observed closed arm entries revealed that the Coc10/MS group made significantly more closed arm entries at baseline when compared to the Sal/MS group (Coc10/MS: 2.92 ± 1.57 vs. Sal/MS: 5.87 ± 1.95, $p<0.0001$). Coc10/MS had no significant difference in closed arm entries at baseline when compared to both the Coc10/No MS group and the Sal/No MS groups individually. The Sal/MS made significantly more closed arm entries when compared to the Coc10/No MS group at baseline (Sal/MS: 5.86 ± 1.95 vs. Coc10/No MS: 3.13 ± 1.06, $p<0.0001$). Sal/MS also had significantly more closed arm entries than the Sal/No MS group during the baseline test (Sal/MS: 5.86 ± 1.95 vs. Sal/No MS: 4.00 ±

1.28, $p<0.05$). There were no significant between group comparisons in the the number of closed arm entries made during the post-injection trial. The post-extinction trial revealed no significant difference between the Coc10/MS group and both the Sal/MS group and the Coc10/No MS group. The Coc10/MS group did however present significantly fewer closed arm entries than the Sal/No MS group during the post-extinction timepoint (Coc10/MS: 1.5 ± 0.52 vs. Sal/No MS: 3.41 ± 1.78, $p<0.005$). Sal/MS had significant differences in closed arm entries with neither the Coc10/No MS group nor the Sal/No MS after the extinction period. The Coc10/No MS group had significantly fewer closed arm entries than the Sal/No MS group after the extinction period (Coc10/No MS: 1.06 ± 0.46 vs. Sal/No MS: 3.41 ± 1.78, $p<0.0001$) (See Fig. 4).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of protected head dips (PHD) performed and a significant difference in both the time point ($F_{2, 126} = 12.81$, $p<0.0001$) and group variables ($F_{3, 126} = 3.29$, $p<0.0001$). Tukey's multiple comparisons testing within each group revealed a significant increase in PHD observations from the baseline test to the post-extinction test in the Coc10/MS group (Baseline: 3.17 ± 2.25 vs. Post-Extinct: 6.58 ± 3.45, $p<0.01$). Neither the baseline nor the final timepoints for the Coc10/MS group differed in the number of PHDs observed when compared to the post-injection test. The Sal/MS group showed a significant increase from baseline to post-injection (Baseline: 2.57 ± 1.39 vs. Post-inj: 6.71 ± 3.10, $p<0.05$), but had no significant differences when baseline was compared to the post-extinction test and the post-injection test was compared to the post-extinction test. The Coc10/No MS group experiences no significant changes in the number of PHDs expressed throughout each of the three trials in the EPM. The Sal/No MS group had a significant increase in PHDs observed at baseline compared to both the post-injection time and

the post-extinction time (Baseline: 4 ± 1.65 vs. Post-inj: 7 ± 2.76 vs. Post-Extinct: 6.92 ± 3.29, $p<0.05$ for both comparisons). There was no significant difference in the number of PHDs observed from post-injection to post-extinction in the Sal/No MS group.

Between group comparisons revealed no significant differences in PHDs observed between any of the groups at baseline. There was also no significant difference between any of the groups in the number of PHDs made during post-injection testing. The Coc10/MS and Sal/MS group had no significant difference in the number of PHDs observed after extinction. Coc10/MS did perform significantly more PHDs than the Sal/MS group after the extinction period (Coc10/MS: 6.58 ± 3.45 vs. Sal/MS: 3.53 ± 2.75, $p<0.05$). No significant differences in PHD expression were found between the Coc10/MS group and the Sal/No MS group during the post-extinction test. The Sal/MS group had no significant differences in PHDs after extinction with both the Coc10/No MS group and the Sal/No MS group. The Coc10/No MS made significantly fewer PHDs than the Sal/No MS group (Coc10/No MS: 3.53 ± 2.75 vs. Sal/No MS: 6.92 ± 3.29, $p<0.01$) (See Fig. 5) (See Fig. 5).

Two-way ANOVA analysis (timepoint x group) showed a significant main effect ($F_{6, 126} = 5.43$, $p<0.0001$) for the number of unprotected head dips (UHD) performed and a significant difference in both the time point ($F_{2, 126} = 21.76$, $p<0.0001$) and group variables ($F_{3, 126} = 8.02$, $p<0.0001$). Within group multiple comparisons testing revealed a significant decrease in the number of UHD's made by the Coc10/MS group when the baseline test was compared to both post-injection and post-extinction tests (Baseline: 10.5 ± 3.60 vs. Post-inj: 4.75 ± 2.76 vs. Post-Extinct: 2.17 ± 1.95, $p<0.0001$ for both comparisons). The Coc10/MS experienced no significant change in UHDs from the post-injection time to the post-extinction. There was no

significant change in the number of UHDs performed by the Sal/MS group throughout their series of tests. The Coc10/No MS group experienced a significant decrease in UHDs from post-injection testing to post-extinction testing (Baseline: 5.47 ± 2.95 vs. Post-inj: 3.67 ± 2.80 vs. Post-Extinct: 1.27 ± 1.83, $p<0.0005$ and $p<0.05$ respectively), but there was no significant difference from baseline to post-injection. The Sal/No MS group experienced a decrease from baseline to post-injection (Baseline: 7.17 ± 2.29 vs. Post-inj: 4.33 ± 2.10, $p<0.05$), but experienced no significant change from baseline to post-extinction or post-injection to post-extinction.

Between group comparisons revealed that Coc10/MS made significantly more UHDs at baseline than the Sal/MS group (Coc10/MS: 10.50 ± 3.61 vs. Sal/MS: 5.29 ± 2.81, $p<0.001$),; significantly more UHDs than the Coc10/No MS group at baseline (Coc10/MS: 10.50 ± 3.61 vs. Coc10/No MS: 5.47 ± 2.94, $p<0.0001$; and significantly more UHDs than the Sal/No MS group at baseline (Coc10/MS: 10.50 ± 3.61 vs. Sal/No MS: 7.17 ± 2.29, $p<0.001$). Sal/MS had significant differences with neither Sal/No MS nor Coc10/No MS at baseline. Coc10/No MS and Sal/No MS also did not differ significantly at baseline. There were no significant differences in UHDs between any of the groups during the post-injection EPM trials. After the extinction period, it was revealed that Coc10/MS did not differ significantly from both the Sal/MS group and the Cco10/MS group. Coc10/MS did however show significantly fewer UHD than the Sal/No MS group (Coc10/MS: 2.17 ± 1.94 vs. Sal/No MS: 6.67 ± 4.40, $p<0.001$). The Sal/MS group showed no significant difference in observed UHDs compared to both the Coc10/No MS groups and the Sal/No MS groups. The Coc10/No MS group expressed significantly fewer

UHDs than the Sal/No MS group (Coc10/No MS: 1.27 ± 1.83 vs. Sal/No MS: 6.67 ± 4.40, $p<0.001$) (See Fig. 6).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of end of arm head dips (EHD) performed, but did have a significant difference in both the time point ($F_{2, 126} = 14.99$, $p<0.0001$) and group variables ($F_{3, 126} = 6.65$, $p<0.0001$). Within group multiple comparisons revealed that the Coc10/MS group expressed significantly less EHD after the baseline test (Baseline: 3.08 ± 1.38 vs. Post-inj: 1.33 ± 1.30 vs. Post-Extinct: 1.00 ± 0.85, $p<0.0001$ for both comparisons), but no significant difference between the post-injection test to the post-extinction test. The Sal/MS group demonstrated a significant decrease from baseline to post-injection (Baseline: 2.71 ± 1.70 vs. Post-inj: 0.42 ± 0.53, $p<0.01$), but then experienced a slight increase from post-injection to post-extinction that resulted in insignificant comparisons of both post-injection to post extinction and baseline to post-extinction. The Coc10/No MS group had no significant differences in EHDs when baseline was compared to post-injection or post-injection was compared to post-extinction, but there was a significant decrease in the number of EHDs from baseline to final (Baseline: 1.80 ± 1.42 vs. Post-Extinction: 0.13 ± 0.35, $p<0.005$). There was also a significant decrease in EHD by the Sal/No MS group from baseline to post-injection (, followed by a slight increase from post-injection to post-extinction that resulted in an insignificant difference between post-injection and post-extinction as well as baseline and post-extinction.

Between subjects comparisons revealed no significant difference in EHDs between any of the groups at baseline. Additionally, there were no significant differences in the number of EHDs expressed during the post-injection round of testing. There were also no significant

differences in expressed EHDs when Coc10/MS was compared to both Sal/MS and Coc/No MS during the post-extinction test. There was a significant difference between the Coc10/MS group and the Sal/No MS group (Coc10/MS: 1.00 ± 0.85 vs. Sal/No MS: 2.75 ± 2.83, $p<0.05$). Sal/MS showed no significant differences in the number of EHDs expressed during the post-extinction trial compared to both Coc10/No MS and Sal/No MS. After extinction Coc10/No MS did show significantly fewer EHDs than Sal/No MS (Coc10/No MS: 0.13 ± 0.35 vs. Sal/No MS: 2.75 ± 2.83, $p<0.0001$) (See Fig. 7).

Two-way ANOVA analysis (timepoint x group) showed a significant main effect for the number of stretch attend postures (SA) performed ($F_{6, 126} = 3.29$, $p<0.005$), but did have a significant difference in both the time point ($F_{2, 126} = 21.11$, $p<0.0001$) and group variables ($F_{3, 126} = 8.45$, $p<0.0001$). Within subjects multiple comparisons revealed that rats in the Coc10/MS group demonstrated a significant increase in SA after the baseline test (Baseline: 1.00 ± 0.60 vs. Post-inj: 4.33 ± 1.72 vs. Post-Extinct: 4.58 ± 2.67, $p<0.0001$ for both comparisons), but had no significant difference between the post-injection and post-extinction tests. Rats in the Sal/MS group showed a significant increase in SA from baseline to post-extinction (Baseline: 0.86 ± 0.90 vs. Post-Extinct: 3.57 ± 1.72, $p<0.01$), but showed no significant change from baseline to post-injection and from post-injection to post-extinction. Rats in the Coc10/MS group demonstrated a significant increase in SA from baseline to post-injection, and a significant decrease from post-injection to post-extinction (Baseline: 1.40 ± 1.24 vs. Post-inj: 3.67 ± 1.84 vs. Post-Extinct: 2.13 ± 1.78, $p<0.001$ and $p<0.05$ respectively). The significant decrease from post-injection to post-extinction resulted in an insignificant difference in the number of expressed SA between the the baseline and post-extinction of the final group. The rats in the

Sal/No MS group showed no significant change in expressed SA throughout their three different stages of EPM testing.

Between group comparisons of SA revealed no significant difference in the number SA between any of the observed groups at the baseline test time. Coc10/ MS did not significantly differ from either the Sal/MS or Coc10/No MS groups in expressed SA, but did show significantly more SA than the Sal/No MS group of rats during post-injection testing (Coc10/MS: 4.33 ± 1.72 vs. Sal/No MS: 2.08 ± 1.83, $p<0.01$). Sal/MS demonstrated no significant difference in SA when compared to both Coc10/MS and Sal/No MS at baseline. Coc10/No MS also showed no significant difference in SA expression when compared to Sal/No MS. During the post-extinction tests, Coc10/MS showed no significant difference in SA performed when compared to Sal/MS, but did show a significantly more SA than the Coc10/No MS group (Coc10/MS: 4.58 ± 2.13 vs. Coc10/No MS: 2.13 ± 1.77, $p = 0.001$). Coc10/MS also demonstrated significantly more SA than Sal/No MS (Coc10/MS: 4.58 ± 2.13 vs. Sal/No MS: 1.08 ± 0.79, $p<0.0001$). Sal/MS did not appear to significantly differ in SA posture compared to Coc10/No MS, but it did perform significantly more SA than Sal/No MS (Sal/MS: 3.57 ± 1.72 vs. Sal No MS: 1.4 ± 1.24, $p<0.01$) during post-extinction testing. There was no significant difference in SA posture between Coc10/No MS and Sal/No MS during post-extinction testing (See Fig. 8).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the percent of time spent in the open (POA), but did have a significant difference in both the time point ($F_{2, 126} = 29.18$, $p<0.0001$) and group variables ($F_{3, 126} = 3.03$, $p<0.05$). Within subjects comparisons revealed a significant decrease in POA from baseline to post-injection, and a

significant decrease in POA from baseline to final (Baseline: 30.47% ± 10.55% vs. Post-inj: 15.89% ± 12.85% vs. Post-Extinct: 8.58% ± 6.23%, $p<0.005$ and $p<0.0001$ respectively) in the Coc10/MS group. Coc10/MS did not have any significant change in POA from post-injection to post-extinction. The Sal/MS group also had a significant decrease in POA after baseline when compared to both post-injection and post-extinction (Baseline:32.76% ± 12.82% vs. Post-inj: 13.57% ± 6.99% vs. Post-Extinct: 13.90% ± 11.10%, $p<0.005$ for both comparisons), but showed no significant change in POA from post-injection to post-extinction. Coc10/No MS also showed a significant decrease in the POA when baseline is compared to both the post-injection and post-extinction times (Baseline: 23.40% ± 12.41% vs. Post-inj: 14.20% ± 9.21% vs. Post-Extinct: 6.87% ± 7.33%, $p<0.05$ and $p<0.0001$ respectively), but showed no significant change from post-injection to post-extinction. The Sal/No MS group had only a significant decrease in POA from baseline to post-injection testing (Baseline: 26.36% ± 8.21% vs. Post-inj: 16.08% ± 8.56%, $p<0.0001$), with no change in POA from baseline to post-extinction or from post-extinction from post-extinction.

Between group comparisons revealed no significant differences in POA between any of the tested groups at both the baseline and post-injection time points. There was no significant difference in POA between Coc10/MS and Sal/MS, as well as a lack of difference between Coc10/MS and Coc10/No MS. There was, however, significantly lower POA during the post-extinction round of EPM testing in Coc10/MS when compared to Sal/No MS (Coc10/MS: 8.58% ± 6.23% vs. Sal/No MS: 21.47% ± 12.92%, $p<0.05$). Sal/MS showed no significant difference in POA after extinction when compared to both Coc10/No MS and Sal/No MS. Coc10/No MS expresses a significantly lower POA when compared to Sal/No MS for the

post-extinction stage of testing (Coc10/No MS: 6.86% ± 7.32% vs. Sal/No MS: 21.47% ± 12.92%, $p<0.05$) (See Fig. 9).

Two-way ANOVA analysis (timepoint x group) showed a significant main effect for the percent of time in the open field spent at the edge (PAE) performed ($F_{6, 126} = 4.42$, $p<0.0005$), and did have a significant difference in both the time point ($F_{2, 126} = 20.92$, $p<0.0001$) and group variables ($F_{3, 126} = 39.50$, $p<0.0001$). Within group multiple comparison revealed that the Coc10/MS group demonstrated no significant change in PAE throughout the three different times of OF testing. The Sal/MS group showed no significant difference when the baseline test was compared to the post-injection test, but did have a significant increase in PAE after the end of their extinction period compared to both baseline and post-extinction (Baseline:40,24% ± 26.55% vs. Post-inj: 35.00% ± 34.32% vs. Post-Extinct: 88.67% ± 8.62%, $p<0.005$ for both comparisons) Rats in the Coc10/No MS showed no significant difference in baseline PAE compared to post-injection PAE, but the baseline and post-injection measurements both were significantly lower than the PAE measured after extinction (Baseline:76.18% ± 14.16% vs. Post-inj: 75.73% ± 13.95% vs. Post-Extinct: 93.36% ± 10.28%, $p<0.0001$ for both comparisons). Sal/No MS showed no significant change in PAE throughout the course of open field testing.

Between subjects comparisons revealed that Coc10/MS has a significantly higher PAE at baseline compared to Sal/MS (Coc10/MS: 90.81% ± 5.05% vs. Sal/MS: 40.24% ± 26.55%, $p<0.0001$). Coc10/Ms also expressed a significantly higher PAE than Coc10/No MS (Coc10/MS: 90.81% ± 5.05% vs. Coc10/No MS: 76.18% ± 14.16%, $p<0.05$) at baseline. Coc/MS expressed no significant difference when compared to Sal/No MS at baseline. Sal/MS expressed significantly lower PAE than Coc10/No MS at baseline (Sal/MS: 40.24% ± 26.55% vs.

Coc10/No MS: 76.18% ± 14.16%, $p<0.05$), as well as significantly lower PAE than Sal/No MS at baseline (Sal/MS: 40.24% ± 26.55% vs. Sal/No MS: 90.61% ± 9.55%, $p<0.0001$). Coc10/No MS also had a significantly lower PAE at baseline than Sal/No MS (Coc10/No MS: 76.18% ± 14.16% vs. Sal/No MS: 90.61% ± 9.55% , $p<0.05$). Immediately after the injection series had ended, the Coc10/MS group showed a significantly higher PAE than the Sal/MS group (Coc10/MS: 83.67% ± 8.40% vs. Sal/MS: 35.00% ± 34.31% , $p<0.0001$), but has no significant differences with both the Coc10/No MS and Sal/No MS groups. Sal/MS showed significantly lower PAE than the Coc10/No MS group (Sal/MS: 35.00% ± 34.31% vs. Coc10/No MS: 75.73% ± 13.94% , $p<0.0001$), as well as a significantly lower PAE tha the Sal/No MS group (Sal/MS: 35.00% ± 34.31% vs. Sal/No MS: 82.69% ± 12.38% , $p<0.0001$) during the post-injection testing. There was no significant difference between the PAEs of the Coc10/No MS and the Sal/No MS groups. There were no significant differences in PAE between any of the test groups for the post-extinction OF test time point (See Fig. 10).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the percent of time in number of squares crossed in the open field (SC), but did have a significant difference in both the time point ($F_{2, 126} = 7.84$, $p<0.001$) and group variables ($F_{3, 126} = 4.46$, $p<0.005$). Within group multiple comparisons revealed no significant difference in the number of SC for the Coc10/MS group when baseline was compared to both post-injection and final, but there was a significant difference in SC when post-injection is compared to post-extinction (Post-inj: 43.83 ± 25.76 vs. Post-Extinct: 12.58 ± 13.29, $p<0.05$). Sal/MS expressed no significant change in SC throughout its three different trials in the OF. Coc10/No MS did not show any significant change in SC when baseline was compared to post-injection, but

post-extinction did show significantly lower SC than both baseline and post-injection (Baseline:44.87 ± 28.53 vs. Post-inj: 44.87 ± 28.53 vs. Post-Extinct: 14.33 ± 26.52 , $p = 0.01$ for both comparisons). There was no significant change in SC for the Sal/No MS group throughout the three different time points in the OF testing.

Between group multiple comparisons revealed that Coc/MS had significantly lower SC at baseline when compared to Sal/MS (Coc10/MS: 20.83 ± 16.28 vs. Sal/MS: 62.57 ± 37.13, $p<0.05$), but was not significantly different than both the Coc10/No MS groups and the Sal/No MS groups at baseline. Sal/MS did not express significantly different SC than the Coc10/No MS group, but did have significantly higher SC than the Sal/No MS group (Sal/MS: 62.57 ± 37.13 vs. Sal/No MS: 20.41 ± 22.52 , $p<0.05$) at baseline. There was no significant difference in SC at baseline between the Coc10/No MS and Sal/No MS groups. Post-injection and post-extinction comparisons between groups revealed no significant differences.

2.2 Second Cohort

Two-way ANOVA analysis (timepoint x group) showed a significant main effect for the number of open arm entries performed (OAE)($F_{6, 105} = 3.31$, $p=0.005$), and did have a significant difference in both the time point ($F_{2, 105} = 14.31$, $p<0.0001$) and group variables ($F_{3, 105} = 11.77$, $p<0.0001$). Within group multiple comparisons revealed that the Coc20/MS group experienced no significant changes in OAE throughout the three different testing times. The Coc20/No MS group showed no significant changes from baseline to post-injection and from post-injection to post-extinction, but did show a significant decrease in OAE from baseline to post-extinction (Baseline: 2.67 ± 1.95 vs. Post-Extinct: 1.07 ± 1.33, $p<0.05$).

Between group multiple comparisons revealed that the Coc20/MS group made significantly fewer OAE than the Sal/MS group (Coc20/MS: 2.60 ± 1.14 vs. Sal/MS: 6.86 ± 2.73, $p<0.0001$), but did not significantly differ from the Coc20/No MS or Sal/No MS during the baseline stage of testing. Rats in the Sal/MS showed significantly more OAE than both the Coc20/No MS (Sal/MS: 6.86 ± 2.73 vs. Coc20/No MS: 2.67 ± 1.95, $p<0.0001$) and Sal/No MS groups (Sal/MS: 6.86 ± 2.73 vs. Sal/No MS: 3.16 ± 1.03, $p<0.0001$) during baseline tests. The Coc20/No MS group and Sal/No MS groups did not significantly differ in the number of OAE observed at baseline. No significant differences in OAE were found between any of the tested groups during the post-injection stage of EPM testing. After the extinction period had ended, the Coc20/MS group was not found to have any significant differences in OAE compared to the Sal/MS, Coc20/No MS, and Sal/no MS groups. Rats in the Sal/MS groups also showed no significant differences in OAE compared to the Coc20/No MS and Sal/No MS during post-extinction testing. The Coc20/No MS group showed significantly fewer OAE than the Sal/No MS group (Coc20/No MS: 1.07 ± 1.34 vs. Sal/No MS: 2.75 ± 1.42, $p<0.05$) during the post-extinction stage of EPM testing (See Fig. 12).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of open arm entries performed (CAE), but did have a significant difference in both the time point ($F_{2, 105} = 9.99$, $p=0.0001$) and group variables ($F_{3, 105} = 7.91$, $p<0.0001$). Within group multiple comparisons revealed no significant differences in expressed CAE between any of the testing times for the Coc20/MS group. Coc20/No MS also showed no significant change in expressed CAE throughout the three testing points

Between group testing revealed that the rats in the Coc20/MS group performed significantly fewer CAE than the Sal/MS group (Coc20/MS: 2.60 ± 1.67 vs. Sal/MS: 5.87 ± 1.95, $p<0.005$), but did not differ significantly from both the Coc20/No MS group and Sal/No MS group during the baseline test. Rats in the Sal/MS group performed significantly more CAE than the Coc20/No MS group (Sal/MS: 5.87 ± 1.95 vs. Coc20/MS: 2.80 ± 2.04 , $p<0.0005$), but showed no significant difference when compared to the Sal/No MS group at baseline. Coc20/No MS also showed no significant difference with the Sal/No MS group during baseline EPM testing. The post-injection round of testing revealed no significant differences in expressed CAE between any of the tested groups. Rats in the Coc20/MS group did not significantly differ in their expression of CAE from the Sal/MS, Coc20/NoMS, and Sal/No MS groups after the full extinction period. The Sal/MS group also did not significantly in CAE differ from both the Coc20/No MS group and the Sal/No MS group during post-extinction EPM testing. Coc20/No MS expressed significantly fewer CAE than the Sal/No MS group during post-extinction testing (Coc20/MS: 1.60 ± 0.91 vs. Sal/MS: 4.42 ± 1.78, $p<0.005$) (See Fig. 13).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of protected head dips performed (PHD), but did have a significant difference in both the time point ($F_{2, 105} = 6.87$, $p<0.005$) and group variables ($F_{3, 105} = 17.96$, $p<0.0001$). Within group multiple comparisons revealed that the Coc20/MS group did not significantly change the number of PHDs expressed during each of the three testing times. The Coc20/No MS group showed an increase in the number of observed PHDs when the baseline test is compared to post-injection testing (Baseline: 1.40 ± 1.40 vs. Post-Injection: 3.6 ± 2.41, $p<0.05$).

Between group multiple comparisons revealed that the Coc20/MS group did not express a significantly different number of PHD compared to the Sal/MS, Coc20/No MS, and the Sal/No MS groups at the baseline stage of testing. Rats in the Sal/MS groups also performed no significant difference of PHDs when compared to both the Coc20/No MS and the Sal/No MS groups at baseline. Coc20/No MS, however, did show fewer PHDs when the compared to the Sal/No MS group at baseline. During the post-injection EPM testing, Coc20/MS showed significantly fewer PHDs than the Sal/MS (Coc20/MS: 1.80 ± 2.05 vs. Sal/MS: 6.71 ± 2.62, $p<0.01$) and Sal/No MS groups (Coc20/MS: 1.80 ± 2.05 vs. Sal/No MS: 7.00 ± 2.76, $p<0.01$), but did not significantly differ in PHD expression compared to the Coc20/No MS group at post-injection. Rats in the Sal/MS group performed significantly more PHDs than the Coc20/No MS group during post-injection testing (Sal/MS: 6.71 ± 2.62 vs. Coc20/No MS: 3.6 ± 2.41, $p<0.05$), but did not show a significantly different number of PHDs compared to the Sal/No MS group. Coc20/No MS showed no significant difference in PHD expression compared to the Sal/No MS group during post-injection EPM testing. After the extinction period a Coc20/MS showed a significantly lower number of PHD's compared to the Sal/MS group (Coc20/MS: 1.00 ± 2.73 vs. Sal/MS: 5.42 ± 3.10, $p<0.05$) and the Sal/No MS group (Coc20/MS: 1.00 ± 2.73 vs. Sal/No MS: 6.92 ± 3.28, $p=0.0001$), but showed no significant difference with the Coc20/No MS group. The Sal/MS group showed no significant differences in PHDs performed with both the Coc20/No MS and the Sal/No MS groups during the post-extinction EPM tests. Rats in the Coc20/No MS group performed significantly fewer PHDs than the Sal/No MS group (Coc20/No MS: 2.73 ± 2.79 vs. Sal/No MS: 6.92 ± 3.28, $p<0.0005$) (See Fig. 14).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of unprotected head dips performed (UHD), but did have a significant difference in both the time point ($F_{2, 105} = 9.07$, $p<0.005$) and group variables ($F_{3, 105} = 7.77$, $p<0.0001$). Within group comparisons revealed that the Coc20/MS group expressed a significant decrease in UHDs after baseline, but did change significantly from post-injection to post-extinction (Baseline: 5.40 ± 2.95 vs. Post-inj: 3.67 ± 2.32 vs. Post-Extinct: 1.27 ± 1.84, $p<0.05$ for both comparisons). The Coc20/No MS expressed no significant difference of UHDs from baseline to post-injection and from post-injection to final, but did show a significant decrease from baseline to post-extinction (Baseline: 5.27 ± 3.15 vs. Post-Extinct: 2.26 ± 2.34, $p<0.05$).

Between group comparisons revealed no significant differences in UHDs between any of the tested groups during baseline EPM trials. There were also no significant differences in observed UHDs between any of the tested groups during the post-injection EPM trials. Coc20/MS did not show a significantly different number of UHDs when compared to both the Sal/MS and Coc20/No MS groups, but did express significantly fewer UHDs than the Sal/No MS group (Coc20/MS: 1.20 ± 2.27 vs. Sal/No MS: 6.67 ± 4.40, $p<0.005$). Sal/MS did not significantly differ in expressed UHD during the post-extinction EPM trial compared to both the Coc20/No MS and Sal/No MS groups. The Coc20/No MS group showed significantly fewer UHDs than the Sal/No MS group during the post-extinction test (Coc20/No MS: 2.67 ± 2.34 vs. Sal/No MS: 6.67 ± 4.40, $p<0.001$) (See Fig. 15).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect and no significant timepoint effect for the number of end of arm head dips performed (EHD), but did have a significant difference in both the and group variables ($F_{3, 105} = 5.58$, $p<0.005$). Within

group multiple comparisons revealed that both the Coc20/MS and Coc20/No MS Groups did not significantly differ in performed EHDs throughout their various EPM trials.

Between group multiple comparisons revealed that Coc20/MS did not significantly differ from the rats in the Sal/MS, Coc20/No MS, and the Sal/No MS groups in the number of EHDs observed during baseline. The Sal/MS group also demonstrated no significant differences with the Coc/No MS and the Sal/No MS when comparing the number of EHDs observed at baseline. The Coc20/No MS did, however, show a significantly lower number of EHDs than Sal/No MS at baseline (Coc20/No MS: 1.00 ± 1.25 vs. Sal/No MS: 3.00 ± 1.53, $p<0.05$). No significant differences in EHD expression were uncovered between any of the tested groups during the post-injection stage of testing. The Coc20/MS group did not significantly differ in EHD expression from either the Sal/MS or Coc20/No MS groups during post-extinction testing, but did show significantly fewer EHDs than the Sal/No MS group (Coc20/MS: 0.20 ± 1.2 vs. Sal/No MS: 2.76 ± 2.83, $p<0.05$). No significant difference in EHDs was observed when Sal/MS was compared to both the Coc20/No MS and Sal/No MS groups during post-extinction testing. The Coc20/No MS group also did not express a significantly different number of EHDs than the Sal/No MS group (See Fig. 16).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect for the number of stretch attend postures performed (SA), but did have a significant difference in both the time point ($F_{2, 105} = 7.17$, $p<0.005$) and group variables ($F_{3, 105} = 4.22$, $p<0.01$). Within group multiple comparisons revealed no significant change in SA performed by the Coc20/MS group between the baseline, post-injection, and post-extinction testing times. The Coc/No MS group

also showed no significant difference in the SA expressed between each of the three time point levels.

Between group comparisons revealed that the number of SA performed in baseline tests did not significantly differ between any of the tested groups. EPM testing at the post-injection level also showed no significant differences in SA between any of the tested groups. Post-extinction testing revealed no significant difference in performed SA between the Coc20/MS group and the Sal/MS, Sal/No MS, and Coc20/No MS groups. Sal/MS did show significantly more SA behavior than the Coc20/No MS group (Sal/MS: 3.57 ± 2.67 vs. Coc20/No MS: 1.40 ± 1.24, $p<0.01$) during post-extinction EPM testing. No significant difference between the Coc20/No MS and Sal/No MS group could be found from the SA measured during post-extinction testing (See Fig. 17).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect percent of total time in the EPM spent in the open arm (POA) and no significant difference in the group variable, but did have a significant difference in the time point variable ($F_{2, 105} = 13.34$, $p<0.0001$). Within group comparisons revealed that Coc20/MS POA significantly decreased from the baseline test to the post-injection test (Baseline: 43.26% ± 36.02% vs. Post-inj: 13.73% ± 16.40%, $p<0.05$), but saw no significant differences between baseline compared to post-extinction and post-injection compared to post-extinction. The Coc20/No MS group experienced a significant decrease in POA from baseline testing to post-injection testing (Baseline: 47.78% ± 34.00% vs. Post-inj: 22.33% ± 25.08% vs. Post-extinct: 16.40% ± 18.48% , $p<0.05$), and then did not significantly change from post-injection testing to post-extinction testing.

Between groups multiple comparisons revealed that Coc20/MS did not show any significantly different POA from the Sal/MS, Coc20/No MS, and Sal/No MS groups at the baseline level. Sal/MS also did not significantly differ from Coc20/No MS at the baseline time point. Coc20/No MS had a significantly higher PAE than the Sal/No MS group during baseline testing (Coc20/No MS: 47.78% ± 34.00% vs. Sal/No MS: 26.36% ± 8,21%, $p<0.05$). No significant POA differences were found between any of the tested groups during the post-injection and post-extinction EPM time points (See Fig. 18).

Two-way ANOVA analysis (timepoint x group) showed a significant main effect for the percent of total time spent in the open field spent at the edge (PAE)($F_{6, 105} = 6.76$, $p<0.0001$), and a significant difference in the group variable, but no significance in the time point variable ($F_{3, 105} = 14.61$, $p<0.0001$). Within group testing revealed a significant increase in PAE by the Coc20/MS group from baseline to post-extinction (Baseline: 52.07% ± 29.86% vs. Post-inj: 93.93% ± 5.99% vs. Post-extinct: 88.67% ± 8.62% , $p<0.005$ and $p<.05$ respectively), followed by an insignificant decrease in the post-extinction trial. Rats in the Coc20/MS group showed no significant change in PAE from baseline to post-injection and from post-extinction, but revealed a significant decrease in PAE overall when baseline is compared to post-extinction (Baseline: 82.76% ± 14.17% vs. Post-extinct: 59.98% ± 35.44% , $p<0.01$).

Between group multiple comparisons revealed that Coc10/MS did not show significantly different PAE from Sal/MS at baseline, but Coc20/MS did express significantly less PAE than both Coc20/No MS (Coc20/MS: 52.07% ± 29.96% vs. Coc20/No MS: 82.76% ± 14.17%, $p<0.05$) and Sal/No MS at baseline (Coc20/MS: 52.07% ± 29.96% vs. Sal/No MS: 90.61% ± 9.54%, $p<0.005$). Sal/MS showed a significantly lower PAE than Coc20/No MS at the baseline

level of testing (Sal/MS: 40.24% ± 26.55% vs. Coc20/No MS: 82.76% ± 14.17%, $p<0.0001$). Coc20/No MS did not significantly differ in measured PAE with the Sal/No MS group at baseline. The PAE measured at the post-injection time point for the Coc20/MS was significantly higher than Sal/MS (Coc20/MS: 93.93% ± 5.99% vs. Sal/MS: 35.00% ± 34.32%, $p<0.005$) , but did not significantly differ from either the Coc20/No MS group or the Sal/No MS group. Sal/MS significantly differed from Coc20/No MS at the post-injection time point (Sal/MS: 35.00% ± 34.32% vs. Coc20/No MS: 70.46% ± 12.37%). The Coc20/No MS group did not express a significantly different PAE from the Sal/No MS at the post-injection time point. After rats in the test groups experienced their extinction, the PAE of Coc20/MS was determined to be insignificantly different from both Sal/MS and Sal/No MS, but was significantly higher than the Coc20/No MS group(Coc20/MS: 86.67% ± 8.62% vs. Coc20/No MS: 59.98% ± 35.44%, $p<0.05$). Sal/MS was observed expressing a PAE that was insignificantly different that that of Coc20/No MS after extinction. Coc20/No MS had a significantly higher PAE than Sal/No MS after extinction(Coc20/No MS: 59.98% ± 35.44% vs. Sal/No MS: 24.75% ± 22.52% , $p<0.005$) (See Fig. 19).

Two-way ANOVA analysis (timepoint x group) showed no significant main effect and no significant timepoint effect for the number of squares crossed in the open field (SC), but did have a significant difference in both the and group variables ($F_{3, 105}=6.65$, $p<0.0005$). Within group comparisons revealed no significant differences for any of the tested groups between any of the different time points. Between group comparisons showed Coc20/MS showed insignificantly different SC from Sal/MS, Coc20/No MS and Sal/No MS for the baseline time point. Sal/MS showed significantly greater SC (Sal/MS: 62.57 ± 37.13 vs. Coc20/No MS: 17.4 ± 13.99,

$p<0.005$) at the baseline testing time. Analysis of post-injection SC revealed that Coc20/MS performed significantly fewer SC than the Sal/MS group (Coc20/MS: 11.00 ± 9.70 vs. Sal/MS: 57.42 ± 36.66, $p<0.05$). Sal/MS performed significantly more SC than the Coc20/No MS group during the post-injection OF testing time (Sal/MS: 57.42 ± 36.66 vs. Coc20/No MS: 30.47 ± 28.09, $p<0.05$). No significant differences in SC could be found between any of the groups after the extinction period (See Fig. 20).

Discussion

Assessment Maternal Separation

While MS is a commonly used model of early life stress and has been implemented in numerous anxiety studies, in the current study model produced varying levels of baseline anxiety. This is indicated most clearly by the observed differences between all of the MS and No MS groups at the baseline measurement time. At this stage of testing, none of the rats had been exposed to any additional stressors other than maternal separation, and ideally that would indicate that the only differences in behavioral measurements would be between groups that have and have not experienced MS during their upbringing. With this in mind, the behavioral data presented in this study clearly demonstrates a difficulty in establishing a heightened level of baseline anxiety using MS.

The low success of maternal separation in both the first and second testing cohorts is best indicated by the group differences at baseline in the UHD, SA, POA, and SC behavioral measures. Previous studies examining the effects of early life stress on anxiety in rats, as measured by the elevated plus maze and open field test, indicate that rats experiencing MS are less likely to explore the open arms of the EPM and more likely to cross squares in the OF (Jin et

al., 2018). Unprotected head dips are an accurate and easily interpreted measure of open arm exploration because they not only require that the observed rat is out on the open arm, but also require the rat to explicitly exploring the open arm environment by dipping its head below the horizontal plane of the open arm. With this in mind, there would be an expectation that the groups that have experienced MS would show fewer UHD than those who have not. This, however, was not the case in this experiment, as all of the significant measured UHD differences were the result of the Coc10/MS group indicating an unusually high mean UHD, which indicates a very low level of anxiety. The mean UHD was so high that it was significantly different than the Sal/MS group as well as the two groups in the first cohort that did not experience MS. There were no other differences in UHD between groups that did and did not experience MS during pre-weanling, which firmly puts forth the conclusion that the model of MS in this experiment was not successful in establishing basal levels of anxiety.

Stretch attend posture is typically thought to be a measurement of anxiety that differs from behaviors based in open arm because it is considered to be a risk assessment behavior (Albrechet-Souza et al., 2007). Higher levels of observed risk assessment behaviors such as stretch attend indicate a higher level of anxiety, and inversely of UHD, a higher level of SA is expected of rats that had experienced MS in their upbringing compared to group that had not at the baseline EPM measurement. The complete lack of group differences for both cohorts at the baseline EPM test time indicates that MS was not successful in establishing an expressed level of heightened anxiety during the adolescence of the MS groups.

The percent of total time spent in the open arm is one of the most straightforward and reliable behavioral measures of anxiety that can be measured on the EPM. Much like the UHD,

time in the open arm is based on the premise that a rat experiencing more anxiety is less likely to act upon their normal exploratory behavior, and thus well spend less time in the open arm overall. The data collected in this experiment did not indicate any baseline measurements of POA where an MS group spent significantly less time in the open arm than a non-MS group. Due to the reliability and low interpretive margin of error in POA, the possibility of an unsuccessful MS becomes even more likely.

The number of squares crossed during the open field test is the last behavior commonly associated with anxiety in rats that cohesively establishes a failed maternal separation procedure. A study run by Jin et al. explicitly examined the relationship between rats experiencing maternal separation during their upbringing and the behavioral measures that result from that experience. Jin et al.'s study determined that adolescent rats who have been exposed to maternal separation demonstrated significantly higher levels of locomotor activity in an open field test. The manner in which Jin et al. measured locomotor activity differed from this study in that it measured the number of lines crossed at both the edge and in the central zone, making no distinction between the two. With this in mind, the method of measuring locomotor activity has a low validity without a corresponding PAE measurement to support it. The baseline measure in this experiment indicated there was only comparative instance where an MS group crossed significantly more squares than a non-MS group. Inconsistent data such as the MS to non-MS comparison in the number of squares cross during the OF is indicative of the failure of the model of MS used in this experiment to heighten the baseline levels of anxiety in adolescent rats. Without the establishment of a heightened expression of anxiety-like behavior during baseline measurements for rats that have experienced maternal separation, it becomes important to

consider the possibility that any endocrinological changes spurred by MS could be expressed later in adolescence, such as at the post-extinction timepoint. When the same behaviors examined at the baseline time point are explored, this possibility begins to fall short. There was no significant differences between any corresponding MS to non-MS groups, lowering the possibility of a later onset of anxiety-like behavior. Both POA and SC present the same pattern expression in each of their respective tests at the post-extinction time point, which works strongly against the possibility of late behavioral onset. The measurement of stretch attend posture during the post-extinction time period presents the best argument for a late onset because there are two pairs of directly corresponding MS to non-MS groups that significantly differ with the MS groups presenting significantly higher numbers of SA. This behavior alone, however, does not present a convincing argument in favor of an expression of behaviors later in adolescence and appears to be more of an artifact than anything else.

One design aspect of the typical MS model used in early life stress studies that could have been overlooked during this study was the thermoregulation of the pups during the separation. The vast majority of studies examining maternal separation that have shown some significant difference in behavior compared to pups that experienced normal rearing conditions were sure to keep the pups body temperature at a temperature similar to that of the mother (around thirty degrees celsius), but no such thermoregulatory measures were taken in the design of this study. It has been proposed that the mechanisms controlling upregulation of cortico-releasing hormone can be significantly impacted by the presence of a cold-stressor in neonatal rats, but that these mechanisms may be immature during the first postnatal week (Yi and Baram, 1994). This theory could provide an explanation for the lack of consistent anxiety

like behavior expressed by the MS groups of this study. Without an immature mechanism to upregulate CRH due to the cold temperatures from PND 2-7, it is possible that CRH was not elevated appropriately, and therefore the HPA axis would stay relatively unaffected throughout the entire separation period. If this were to be the case, the rats would likely behave in a very similar way to the groups that never experiences separation because they both have unaffected HPA axes. Dysregulation of the HPA axis during the neonatal period is one of the hallmark components of early life stress, and there is not like to be any real change in behavior without such a change.

The lack of an apparent stress response induced by MS could have been the result of dam response to the separation period. The level of dam attentiveness was not monitored or measured in this study, but a heightened level of maternal care immediately following separation has been shown to significantly reduce the stress response of mice later on in life (Own and Patel, 2013). A variable such as this could be another confounding variable of this study that demonstrates the flexibility and complicated nature of stress and anxiety as it presents itself in animal models. Uncontrollable variables such as the mother's response to a separation period is likely one of the primary reason MS struggles with producing consistent stress responses in rats and as result, complicating results produced by studies examining ELS.

Another major limitation in this study was the need to limit the size of a group to the number of pups born into a litter. In order to ensure that the pups were raised in the appropriate maternal care conditions, it was necessary to keep each of the pups in the litter that they were born into. The result of this design was substantial variability between each of the groups, the smallest group having only 5 subjects, and the largest having 15. Groups as small as 5 rats did

not provide an appropriate sample size to produce reliable results within each of the testing conditions. Additionally, the groups in this study most affected by a small sample size were all groups designated to experience MS, which tends to have variable results even without further complications. Smaller groups could have also could have become more resilient to MS because the competition for maternal care was so much lower. Even if there were methodological errors in the design of this studies MS model, smaller groups as Sal/MS (n=7) and Coc20/MS (n=5) could have had a reduced MS affect because the dams of each group were able to provide more attention than the other groups with 12 or more pups.

Assessment of Behavioral Sensitization

The lack of a consistent increase in the locomotor activity measured from the first injection to the seventh injection, to the fourteenth injection indicates that none of the rats exposed to cocaine (Coc10 and Coc20 groups) were sensitized by the end of their injection cycle. Both groups showed significantly higher means of locomotor activity than the saline groups, but the lack of locomotor activity patterns consistent with those seen in previous studies examining behavioral sensitization during adolescence (Elwenspoek et al., 2017) suggests that the rats were missing a hallmark trait of cocaine addiction. The lack of sensitization in the Coc10 groups was originally thought to be attributed to the 10 mg/kg daily dose of cocaine being too low of a dose to induce sensitization as indicated by various studies that injected 10 mg/kg up to three times per day to induce sensitization. This low dosage theory, however, was disproven by the lack of sensitization in the Coc20 groups. A study conducted by Garcia-Rubio et al. suggests that the maternal separation that the rats experiences can reduce the level of locomotor activity expressed by adolescent mice (2016). There would be a possibility the phenomena demonstrated in

Garcia-Rubio's experiment could explain the lack of sensitization in rats exposed to cocaine if not for the fact that both MS and No MS rats exposed to cocaine experienced the same decrease in locomotor activity from the seventh injection to the fourteenth injection.

If this stage of the experiment was to be altered, a binge-pattern of injections would likely produce a more reliable sensitization. The binge-pattern of injections requires that three doses of cocaine be delivered an hour apart shortly after the light portion of the 12h light/dark cycle begins. Establishing behavioral sensitization reliably would have made afforded this study more conclusive results and concrete data analysis.

Another complication of this study that could have affected behavioral sensitization, could be the environmental differences between locomotor activity testing days and normal injection days. Rats in this study were placed back into their respective home cages after every injection other than the days their locomotor activity was monitored. Testing chambers for locomotor activity present an extremely different environment than their home cage (isolation, less bedding), which likely affected their expression of locomotor activity. There is not a lot of empirical evidence to support this idea because the vast majority of behavioral sensitization studies measure locomotor activity on a daily basis. In an ideal situation, locomotor activity would have been monitored on a daily basis, but due to the limitations of an undergraduate schedule, this was not possible.

Assessment of Cocaine-Induced Anxiety

Cocaine has been documented in numerous studies to have prominent influences on the expression of anxiety-like behavior during adolescence, particularly when a period of abstinence is introduced (Valzachi et al., 2013 and El Hage et al., 2012). EPM data from this study indicates

that this trend held true for many of the different groups receiving cocaine injections even though there was no strong behavioral sensitization established. Within group comparisons best illustrate the increase in anxiety-like behavior due to cocaine, which can then be compared to saline groups in the post-injection and post-extinction time points to confirm both that there was a significant change in anxiety from baseline and that the change is likely due to cocaine exposure.

A consistent pattern of increased anxiety-like behavior within cocaine groups is shown by the measurement of UHD, which has previously been established as a reliable measure of anxiety like activity. All groups exposed to cocaine (Coc10/MS, Coc10/No MS, Coc20/MS, Coc20/No MS) all expressed a some significant decrease in UHD after baseline, suggesting an increase in anxiety like behavior. In the case of the of the Coc10/MS and Coc20/MS groups, there was a significant decrease from baseline to post-injection, but no significant change after that. This shared pattern of UHD expression indicates that MS could possibly have some sort of influence on anxiety-like behavior after extinction, which is supported by the pattern of UHD expressed by the Coc10/No MS. The Coc10/MS group expressed no significant decrease in UHD after baseline, but did demonstrate a significant decrease from post-injection to post-extinction. This could indicate that MS blocks some of the additional stress normally caused by an abstinence from cocaine, but this is of course made less clear by both the lack of a similar pattern in the Coc20/No MS group and by the low level of successful MS that has already been established. Although the influence separation has is unclear, groups exposed to only the cocaine stressor (Coc10/No MS and Coc20/No MS) expressed significantly fewer UHD a the post-extinction EPM testing time compared to their direct saline counterparts. This is a very clear indication that the rats exposed to both the low and the high dose of cocaine were more anxious

after a period of abstinence, supporting the findings of previous research to suggest cocaine exposure followed by abstinence has a prominent anxiogenic effect.

Protected head dips also offer some insight into the anxiogenic effects of cocaine exposure and abstinence by demonstrating some of the same comparisons as UHD. PHD can be interpreted in a similar manner as stretch attend posture, that is to say an increase in PHD indicates an increase in anxiety as it is a risk assessment behavior as opposed to an exploratory behavior. The only two cocaine groups that demonstrated a significant increase in PHD after baseline were Coc10/MS and Coc20/No MS. Coc10/MS decreased the number of expressed from baseline to final, which is consistent with the pattern of expression in UHD, but Coc20/MS only demonstrated a significant increase in PHD from baseline to post-injection. Many of cocaine exposed groups demonstrated significant differences from their direct saline counterparts, but with only a significant increase from baseline in the Coc10/MS and Coc20/MS, not many of these significant differences can be attributed solely to cocaine and cocaine abstinence.

Another behavior that provided a seemingly reliable depiction of cocaine's effect on expressed anxiety was POA. Every tested group in both cohorts demonstrated a significant decrease in the POA, suggesting that there was a shared increase in anxiety across all experimental conditions. When groups are compared after extinction, however, both of the Coc10 groups spent significantly less time in the open arm when compared to their saline counterparts, indicating that the lower dose of cocaine resulted in a significant effect whereas the higher dose did not.

Confirming the effect of cocaine and the following period of abstinence becomes more difficult when lack of behavioral sensitization is taken into consideration. Exposure to cocaine and the related abstinence period does seem correlate with the expressed anxiety in some behaviors, but this cannot be determined to be a result of cocaine sensitization due to a lack of sensitization behavior. Anxiety has been well characterized as a symptom of initial cocaine addiction (Sarnyai et al.m 1995), which does suggest that although the rats in this study did not express behavioral sensitization patterns of locomotor activity, they may be experiencing some anxiety related drug-related behavior such as craving. A useful behavioral measurement that could have helped mitigate the impact of such an error would be stereotypy scoring of each rat during each locomotor activity monitoring periods.

Conclusion

This study was limited primarily by scheduling conflicts that come with a full time undergraduate schedule. While it would have been ideal to expose rats to cocaine using the binge pattern of injections and to monitor they stereotypy of each rat during the locomotor activity monitoring, these designs were made nearly impossible by the time constraints. One aspect of anxiety that this study does suggest is the incredible resilience of organisms to endure and overcome stress throughout their lifetime. The half of the rats exposed to MS in this study not only had to endure the stress of an ELS model, but also the exposure to an incredibly addictive substance such as cocaine. After experiencing both of these stressors, these groups of rat often showed no significant difference in anxiety like behavior compared to the control group, truly exemplifying how adaptive a brain can be. Although this study did not find a direct interaction with MS and cocaine addiction due to a myriad of complications, the theories underlying the

study are worthwhile to investigate further. Addiction and anxiety are two interrelated mental health conditions that need to be investigated further in order to better the treatment options of both.

Going forward with this study, other than correcting the possible sources of error in both the maternal separation and cocaine exposure, the study would benefit from a biological assay. One of the more likely targets would be to examine either cortisol or CRH to at each of the behavioral assay testing times. Determining this relationship would allow help definitively confirm if early life stress models such as maternal separation have induced a heightened stress response. Building from this biological assay, a study cocaine self-administration would be useful for determining how early life stress can influence cocaine addiction when the administration is controlled by the rats rather than controlled doses delivered in specific quantities.

Acknowledgements

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Appendix 1: Figures

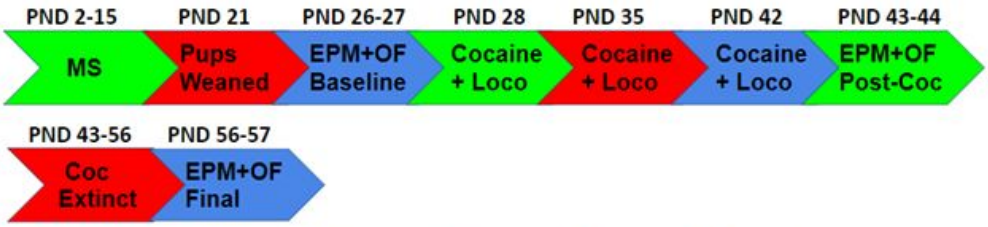

Figure 1. A visual representation of the experimental procedures followed for each group of rats

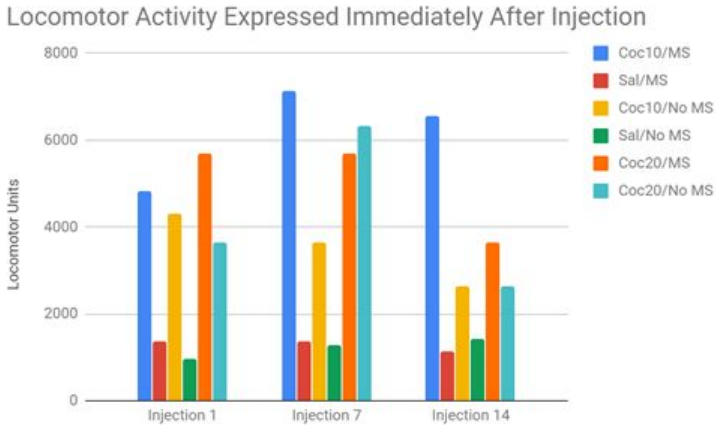

Figure 2. Measurement of locomotor activity for Coc10/MS, Sal/MS, Coc10/No MS, Sal/No MS, Coc20/MS and Coc20/No MS groups at injections 1, 7, and 14 of either cocaine or saline.

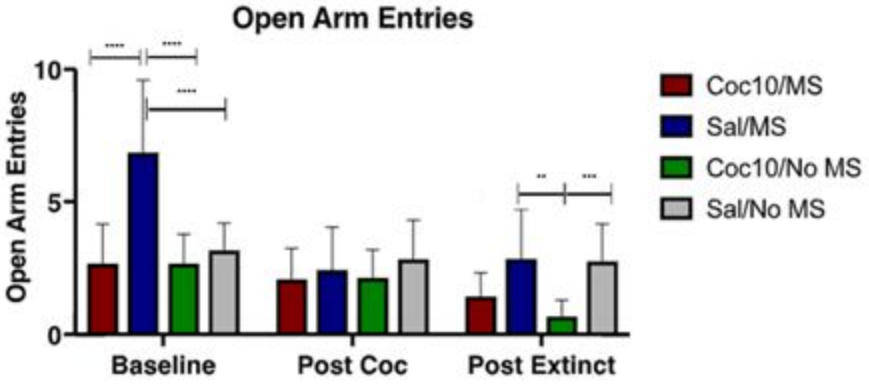

Figure 3. Measurement of the open arm entries performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc10/MS vs. Sal/MS ($p<0.0001$), Sal/MS vs. Coc10/MS ($p<0.0001$), Sal/MS vs. Sal/No MS ($p<0.0001$). Significant differences at post-extinction: Sal/MS vs. Coc10/No MS ($p<0.01$), coc10/MS vs. Sal/No MS ($p<0.001$)

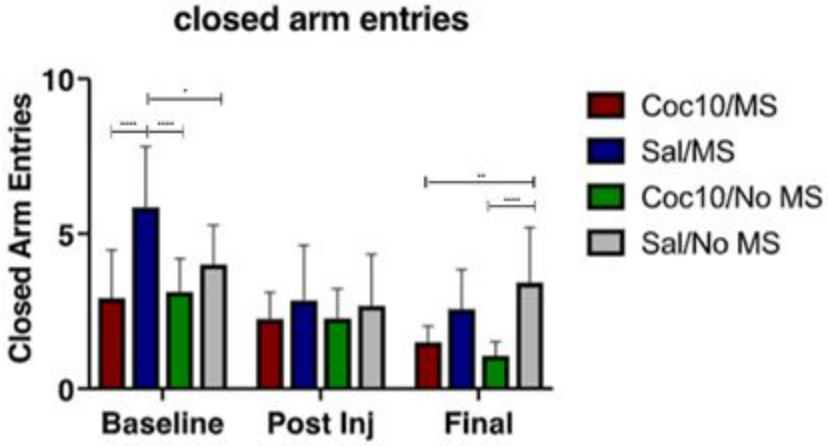

Figure 4. Measurement of the closed arm entries performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc10/MS vs. Sal/MS ($p<0.0001$), Sal/MS vs Coc10/No MS ($p<0.0001$), Sal/Ms vs. Sal/No MS ($p<0.05$). Significant differences at post-extinction: Coc10/MS vs. Sal/No MS ($p<0.01$), Coc10/No MS vs Sal/No MS ($p<0.0001$)

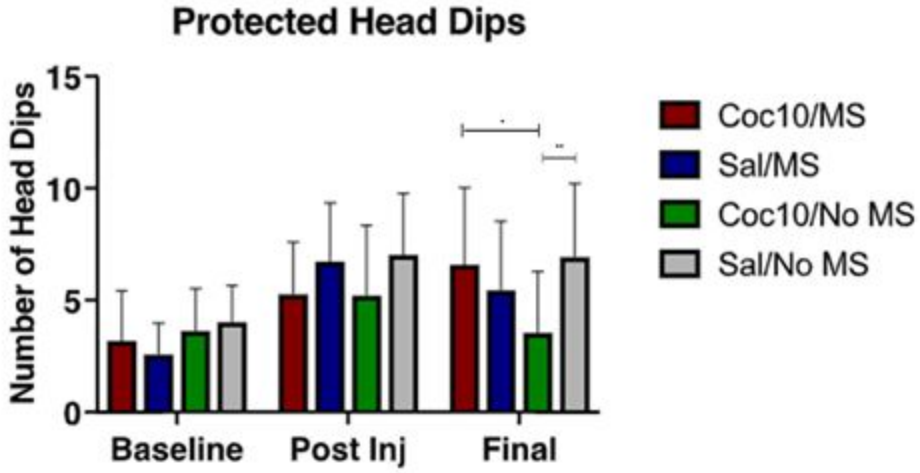

Figure 5. Measurement of the protected head dips performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Coc10/MS vs. Coc10/No MS ($p<0.05$), Coc10/No MS vs. Sal/No MS ($p<0.01$).

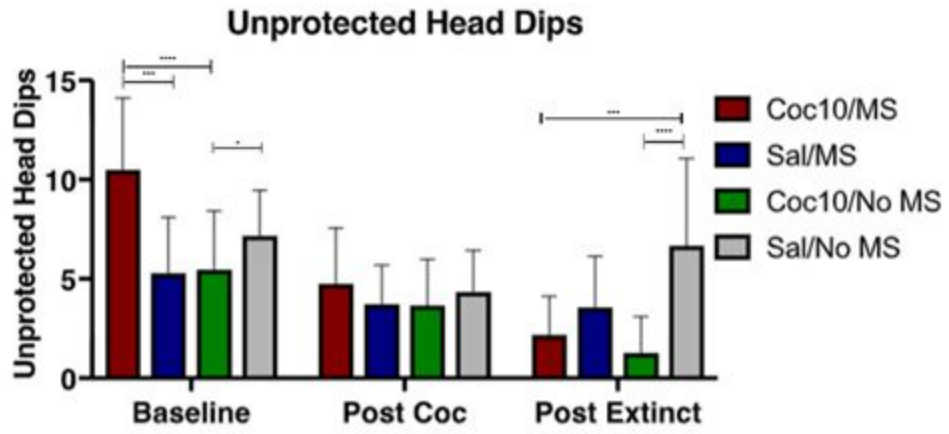

Figure 6. Measurement of the unprotected head dips performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc10/MS vs. Sal/MS ($p<0.001$), Coc10/MS vs. Coc10/No MS ($p<0.0001$), Coc10/No MS vs. Sal/No MS ($p<0.05$). Significant differences at post-extinction: Coc10/MS vs. Sal/No MS ($p<0.001$), Coc10/No MS vs. Sal/No MS ($p<0.0001$).

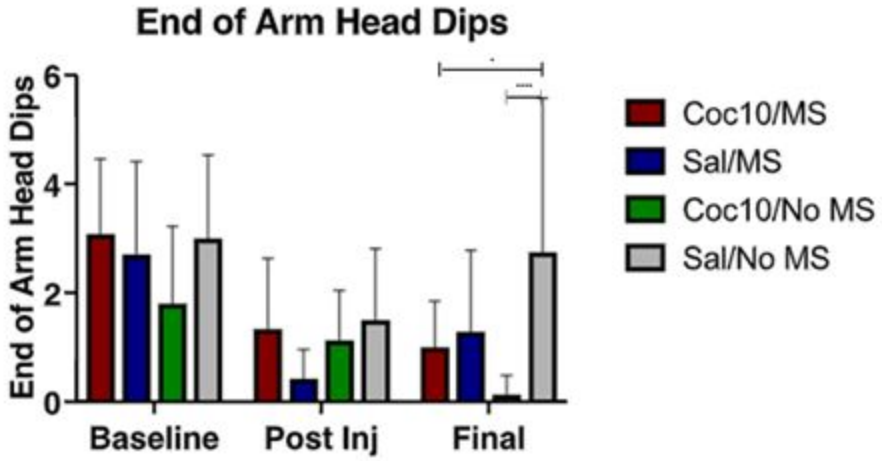

Figure 7. Measurement of the end of arm head dips performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Coc10/MS vs Sal/No MS ($p<0.05$), Coc10/No MS vs. Sal/No MS.

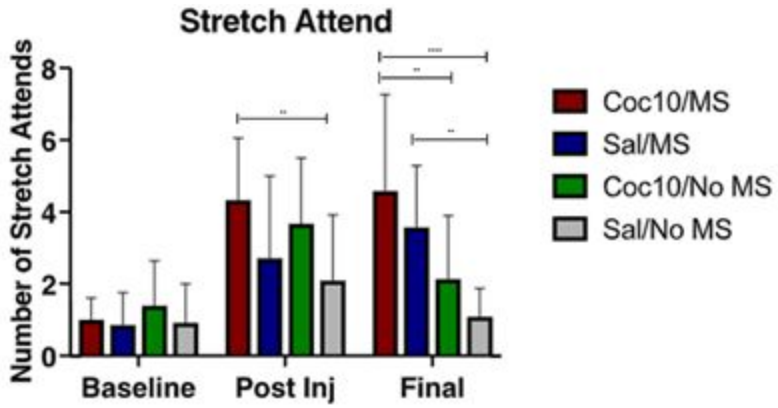

Figure 8. Measurement of the stretch attend posture performed by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-injection: Coc10/MS vs. Sal/No MS ($p<0.01$). Significant differences at post-extinction: Coc10/MS vs. Coc10/No MS ($p<0.01$), Coc10/MS vs. Sal/No MS ($p<0.0001$). Sal/MS vs Sal/No MS ($p<0.01$).

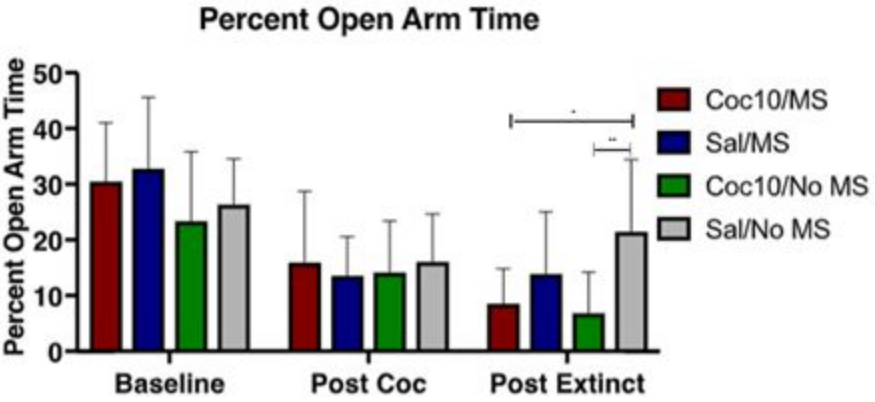

Figure 9. Measurement of the percentage of total time on the EPM spent on the open arm the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Coc10/MS vs. Sal/No MS ($p<0.05$), Coc10/No MS vs. Sl/No MS ($p<0.01$).

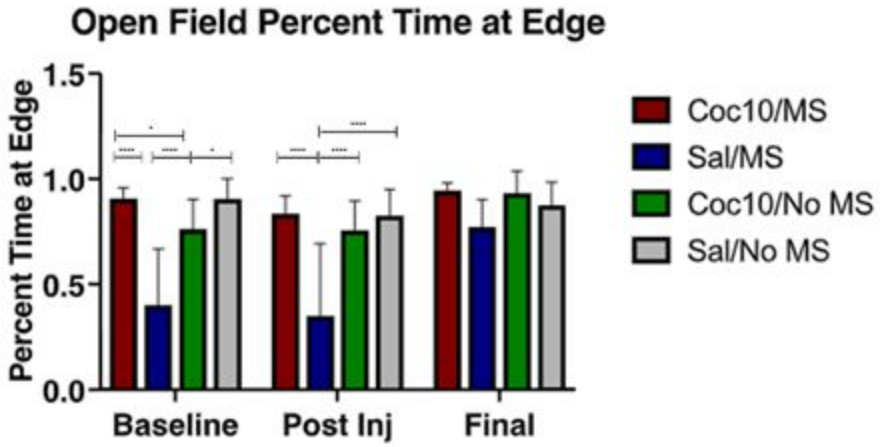

Figure 10. Measurement of the percentage of the total amount of time spent in the OF at the edge by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc10/MS vs. Sal/MS ($p<0.0001$), Coc10/Ms vs. Coc10/No MS ($p<0.05$), Sal/MS vs. Coc10/No MS ($p<0.0001$), Coc10/No MS vs. Sal/No MS ($p<0.05$). Significant differences at post-injection: Coc10/MS vs. Sal/No MS ($p<0.0001$), Sal/MS vs. Coc10/No MS ($p<0.0001$). Sal/MS vs. Sal/No MS ($p<0.0001$).

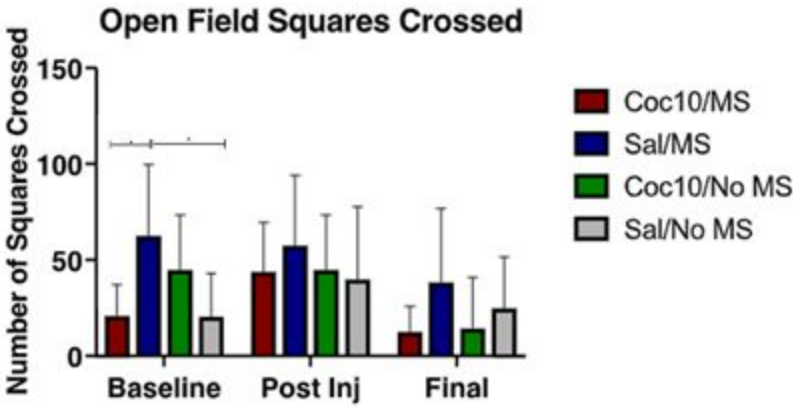

Figure 11. Measurement of the total squares crossed in the OF by the Coc10/MS, Sal/MS, Coc10/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc10/MS vs. Sal/MS ($p<0.05$), Sal/MS vs Sal/No MS ($p<0.05$).

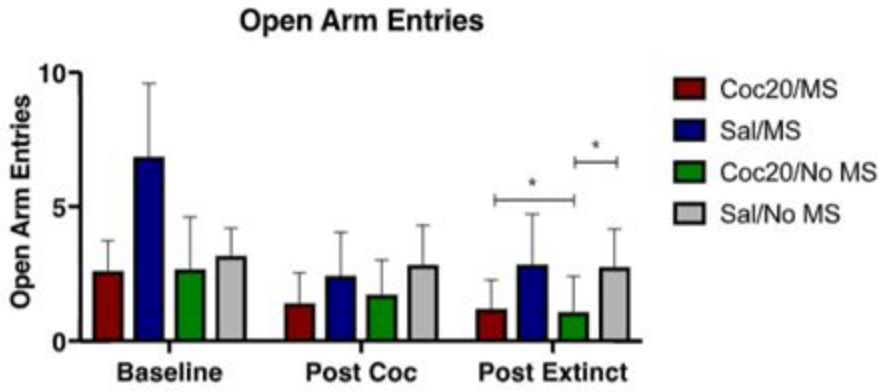

Figure 12. Measurement of the open arm entries performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Coc20/MS vs. Sal/MS ($p<0.05$), Coc20/No MS vs. Sal/No MS ($p<0.05$).

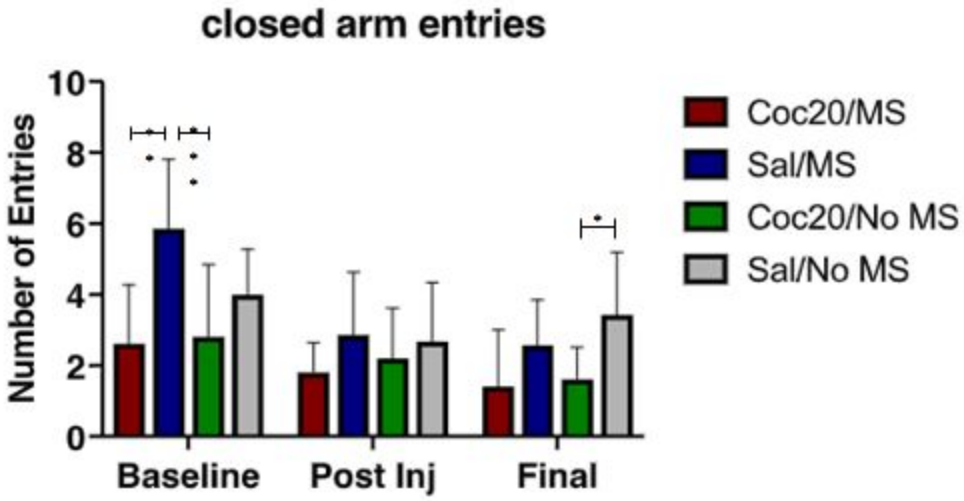

Figure 13. Measurement of the closed arm entries performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc20/MS vs. Sal/MS ($p<0.01$), Sal/MS vs. Coc10/No MS ($p<0.001$). Significant differences at post-extinction: Coc20/No MS vs. Sal/No MS ($p<0.05$).

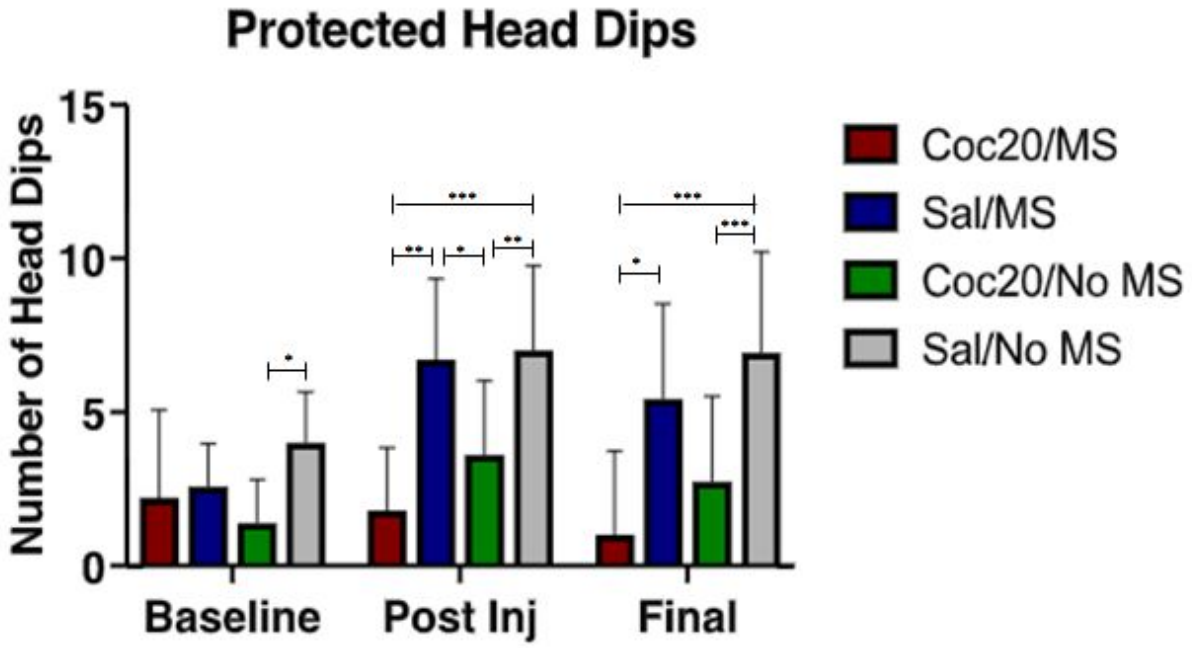

Figure 14. Measurement of the protected head dips performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc20/No MS vs. Sal/No MS. Significant differences at post-injection: Coc20/MS vs. Sal/MS ($p<0.01$), Coc20/MS vs. Sal/No MS ($p<0.001$), Sal/MS vs. Coc20/No MS ($p<0.05$), Coc20/No MS vs. Sal/No MS ($p<0.01$). Significant differences at post-extinction: Coc20/MS vs. Sal/MS ($p<0.05$), Coc20/MS vs. Sal/No MS ($p<0.001$), Coc20/No MS vs. Sal/No MS ($p<0.001$).

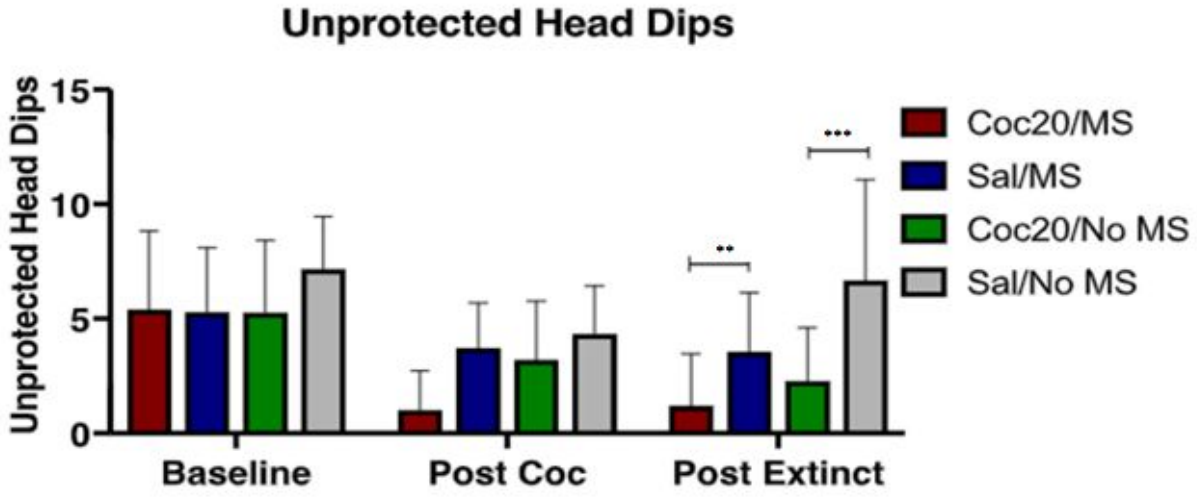

Figure 15. Measurement of the unprotected head dips performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Coc20/MS vs. Sal/MS ($p<0.01$), Coc20/No MS vs. Sal/No MS ($p<0.001$).

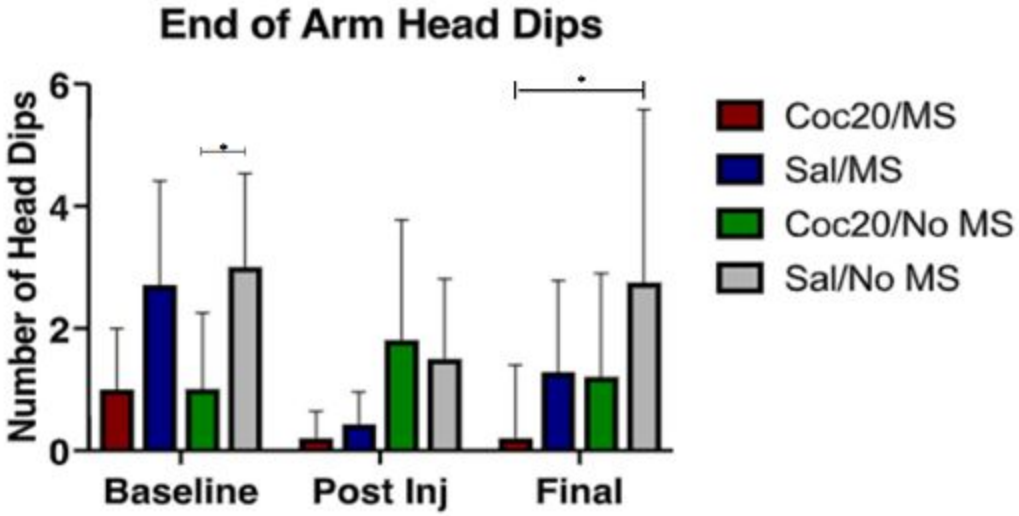

Figure 16. Measurement of the end of arm head dips performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc20/No MS vs. Sal/No MS ($p<0.05$)/ Significant differences at post-extinction: Coc20/MS vs, Sal/No MS ($p<0.05$).

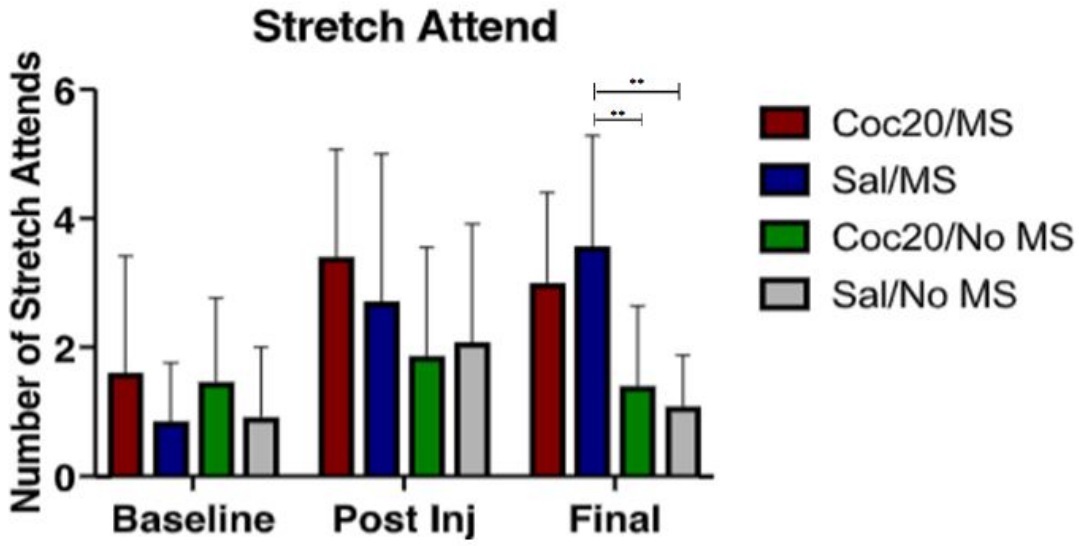

Figure 17. Measurement of stretch attend posture performed by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at post-extinction: Sal/MS vs Coc20/No MS ($p<0.01$), Sal/MS vs Sal/No MS ($p<0.01$).

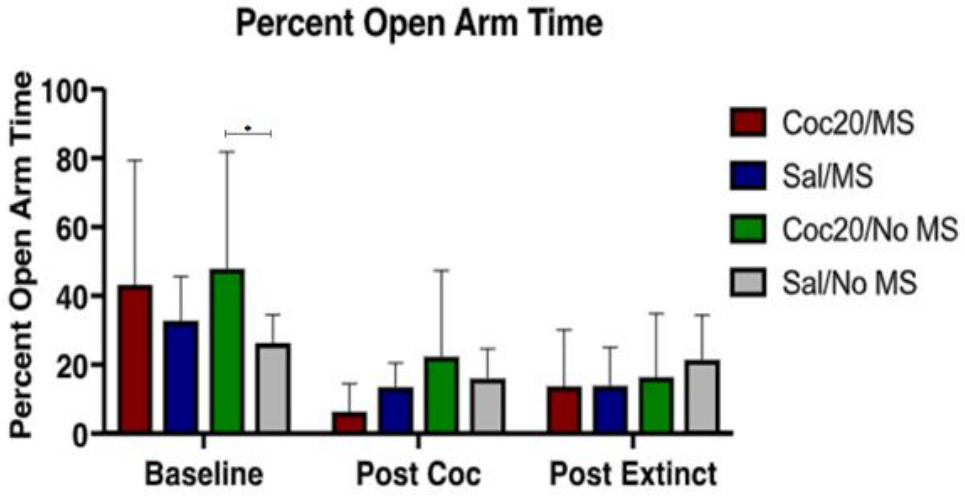

Figure 18. Measurement of percent of total time in EPM spent on the open arm by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant difference at baseline: Coc20/No MS vs. Sal/No MS ($p<0.05$).

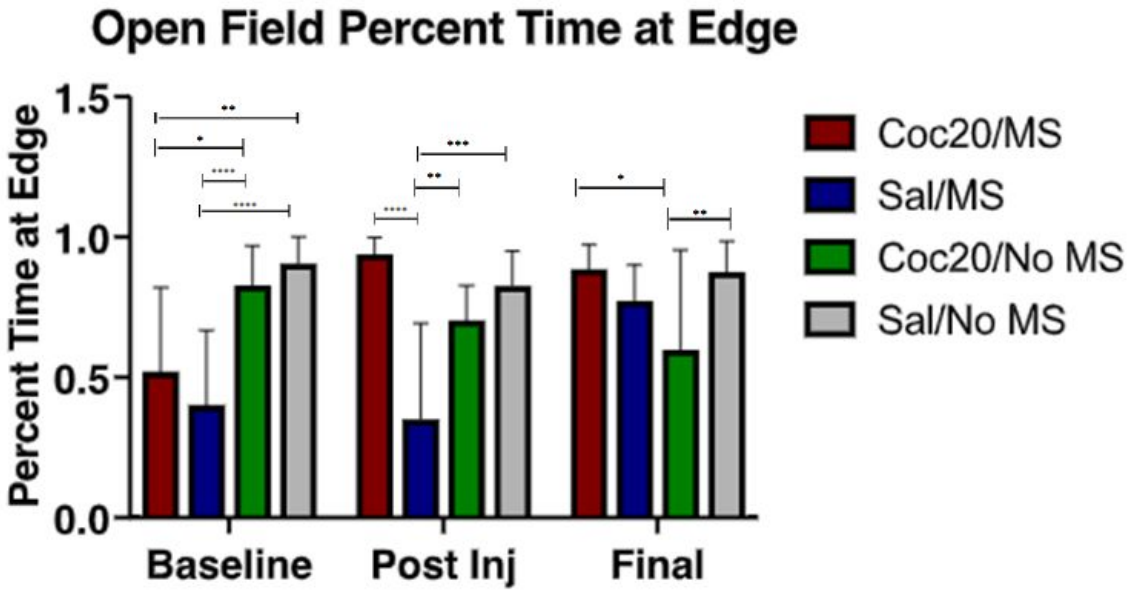

Figure 19. Measurement of percent of total time in OF spent at the edge by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Coc20/MS vs. Coc20/No MS ($p<0.05$), Coc20/Ms vs. Sal/No MS ($p<0.01$), Sal/MS vs. Coc20/MS ($p<0.0001$), Sal/MS vs. Sal/No MS ($p<0.0001$). Significant differences at post-injection: Coc20/MS vs. Sal/MS ($p<0.001$), Sal/MS vs. Coc20/No MS ($p<0.01$), Sal/MS vs. Sal/No MS ($p<0.001$). Significant differences at post-extinction: Coc20/MS vs Coc20/No MS ($p<0.05$), Coc20/No MS vs. Sal/No MS ($p<0.01$).

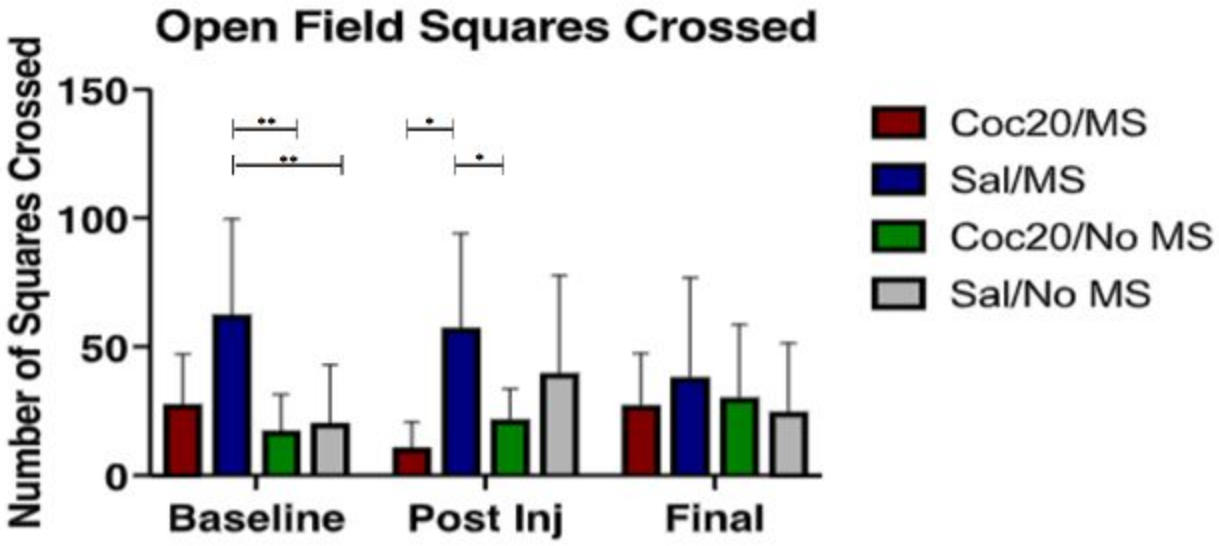

Figure 20. Measurement of total squares crossed in the OF by the Coc20/MS, Sal/MS, Coc20/No MS, and Sal/No MS groups during the Baseline, Post-Injection, and Post-Extinction EPM time points. Significant differences at baseline: Sal/MS vs. Coc20/No MS ($p<0.01$), Sal/MS vs. Sal/No MS ($p<0.01$). Significant differences at post-injection: Coc20/MS vs. Sal/MS ($p<0.05$), Sal/MS vs. Coc20/No MS ($p<0.05$).

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