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Region-Specific Modifications Of Synaptic Proteins In Response To Early-Life Adversity In The Rat Brain

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REGION-SPECIFIC MODIFICATIONS OF SYNAPTIC PROTEINS IN RESPONSE TO EARLY-LIFE ADVERSITY IN THE RAT BRAIN

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DEDICATION

To my darling wife Cristina Hamdan

Thank you for all your love and support, without which I would be lost.

REGION-SPECIFIC MODIFICATIONS OF SYNAPTIC PROTEINS IN RESPONSE TO EARLY-LIFE ADVERSITY IN THE RAT BRAIN

by

JAMEEL NASSER HAMDAN, B.S.

DISSERTATION

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ABSTRACT

Drug addiction is a serious condition affecting approximately 19.7 million people in the United States alone (SAMHSA 2018). Exposure to stress and other adverse conditions has been shown to impact drug-taking behavior and making individuals more vulnerable to addiction. In those already suffering from addiction, abstaining from drug use often gives way to relapse after a stressful event (Lu, Shepard et al. 2003; Shaham, Erb et al. 2000). The experience of adversity during early-life can cause long-lasting changes in the brain, affecting development, behavior, learning and memory, and critical thinking processes, which may persist into adulthood (Aisa, Tordera et al. 2007; Anisman, Zaharia et al. 1998; Banihashemi, O'Neill et al. 2011; Card, Levitt et al. 2005; Hill, Kiss Von Soly et al. 2014; Huot, Plotsky et al. 2002; Kalinichev, Easterling et al. 2002; Kinkead and Gulemetova 2010; Kuhn and Schanberg 1998; Roceri, Hendriks et al. 2002; Romano-Lopez, Mendez-Diaz et al. 2015; Ryan, Musazzi et al. 2009). An overarching goal of our laboratory, and specifically the focus of this dissertation, is to examine the neurological mechanisms that are altered by stress or adversity and promote increased vulnerability to addiction. The goal of this project was to evaluate neurochemical changes in the brain that are caused by early-life adversity, experienced in the form of neonatal maternal separation, and may promote an increased vulnerability to addiction. Our studies incorporated both molecular and behavioral methodologies, comprising the two specific aims of this dissertation. **Specific Aim 1** identifies changes in the expression of addiction-related synaptic proteins in adult male rats with a history of early-life adversity. These animals showed significant changes in the expression of several proteins in four distinct brain regions, as measured by Western blot. **Specific Aim 2** utilizes the behavioral assays of conditioned-place preference to methamphetamine and light-dark box to

assess motivation and anxiety and help elucidate the effects that early-life adversity has on behaviors that are related to drug use. A significant increase in anxiety-like behavior was seen in animals exposed to adversity in early-life, compared to those reared under normal conditions. However, no changes were seen in motivation or response to drug administration in animals that were exposed to adversity early in life. Together, the findings of both Aims of this dissertation demonstrate that early-life adversity can lead to persistent modifications of protein expression in the brain and to altered behavior in adulthood. While we did not observe any changes in the response of our animals to drug application in this paradigm and at the doses tested, other investigators have shown changes in drug-taking behavior in rats exposed to the same neonatal maternal separation model of stress or early-life adversity. The results of the present work include enhanced anxiety-like behavior and modified expression of proteins involved in synaptic function and signaling, both of which may underlie or inform the mechanisms through which increased vulnerability to addiction is mediated in individuals exposed to stress or adversity in early-life. Thus, the goal of this dissertation was to examine how neural and behavioral changes resulting from exposure to early-life adversity contribute to increased vulnerability to addiction.

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GENERAL INTRODUCTION

Adversity is nearly universally experienced by individuals at some time in their life and is defined as a misfortune or difficulty with which one must cope. Physiologically, adversity translates into stress and the activation of bodily stress responses that are mediated through the sympatho-adrenomedullary (SAM; “fight-or-flight”) system and the hypothalamic-pituitary-adrenocortical (HPA) axis. The response to stress or adversity, therefore, is dynamic and can result from stimuli originating from inside and/or outside of the body as well as interactions between these factors (Butler 1993). Stress, in turn, has been implicated in drug use, dependence, and relapse, although the mechanisms through which stress or adversity affects these behaviors are incompletely understood. Exposure to adversity in the form of established stress paradigms such as maternal separation or chronic wheel running has been shown to induce changes in anxiety and drug-taking behavior in mice and rats (Romeo, Mueller et al. 2003; Sobieraj, Kim A Fau - Fannon et al. 2016). It is thought that the long-term effects of stress on drug use, addiction and anxiety are mediated, at least in part, through alterations in dopamine systems in the brain. Support for this comes from the observation that stress-induced or pharmacological modulations of dopaminergic signaling in animal models are able to facilitate the maintenance or reinstatement of methamphetamine use, and increase anxiety-like behavior (Sobieraj, Kim A Fau - Fannon et al. 2016; Zarrindast and Khakpai 2015). Anxiety is a complex psychological state that can be caused by various stressors (Leuner and Shors 2013; Shin and Liberzon 2010). Generally, increased anxiety follows on from stressful or fear-inducing situations, but elevations in anxiety without these external triggers can be indicative of a dysregulation of neuroendocrine regulatory systems and the manifestation of an anxiety disorders (Pêgo, Sousa et al. 2010).

Maternal separation (MS), an established and well-characterized model of early-life adversity (ELA) and stress that is applied in the neonatal period, has been shown to affect neuronal activation, endocrine function, neuronal connectivity, protein expression, and anxiety and fear behaviors in adulthood (Aisa, Tordera et al. 2007; Anisman, Zaharia et al. 1998; Card, Levitt et al. 2005; Huot, Plotsky et al. 2002; Kalinichev, Easterling et al. 2002; Kuhn and Schanberg 1998; Rinaman, Banihashemi et al. 2011; Romeo, Mueller et al. 2003). This strong developmental stress model in rodents mimics the human situation in which an infant has reduced contact with its mother (Vetulani 2013).

In order to determine the effects of MS on protein expression levels in the brain and addiction-related behavior, we evaluated a number of synaptic markers that underlie neuronal signaling in adult male rats that were exposed to MS, compared to control animals that were reared normally. In addition, separate groups of rats undergoing the same treatment regimens were tested for methamphetamine preference under a conditioned place preference (CPP) paradigm, and anxiety-like behavior using a light-dark box test. Although MS is a well-characterized form of ELA, little work has been done to examine its effects on neural plasticity, its potential contribution to increasing addiction vulnerability, or its role in anxiety-like behavior.

1.0 SPECIFIC AIM 1: CHANGES IN PROTEIN EXPRESSION

1.1 ABSTRACT

This aim of the dissertation is to identify changes in protein expression in specific brain regions following ELA in the form of MS. Protein expression levels were determined by Western blot and quantified via densitometry. The animals evaluated in this aim provide a "snapshot" of the neurological condition of the animals that will be used in Aim 2, representing the state of their brain tissue immediately prior to the initiation of the behavioral tests or exposure to methamphetamine. The neural circuits known to be involved in responses to stress and in anxiety- and addiction-related behaviors are extensive, spanning from the cerebral cortex to the medulla and affecting cortical, hippocampal, and other forebrain regions (López, Akil et al. 1999; Pêgo, Sousa et al. 2010). Through the activation of the HPA axis, exposure to adversity and the resulting stress is capable of changing dopaminergic signaling in the brain, leading to changes in several behaviors, including addiction (Herman 2012). Based on these findings, we evaluated dopamine receptor 1 (D_1), dopamine receptor 2 (D_2), dopamine transporter (DAT), tyrosine hydroxylase (TH), *N*-methyl-D-aspartate receptor 1 (NMDAR), post-synaptic density 95 (PSD-95), and α-synuclein protein expression in four brain regions: the medial prefrontal cortex (mPFC), hippocampus (HIP), nucleus accumbens (NAcc), and caudate putamen (CPu). Significant changes in the expression of these molecules are observed in each brain region in ELA rats compared to controls. Taken together, our results suggest that MS is associated with an increase in dopamine-mediated reward, a decrease in spatial learning abilities, and a decrease in decision-making and conditioned learning. As stated previously, MS can induce structural and functional changes in neural, biochemical, and endocrine systems in adult animals (Aisa, Tordera et al. 2007; Anisman,

Zaharia et al. 1998; Card, Levitt et al. 2005; Huot, Plotsky et al. 2002; Kalinichev, Easterling et al. 2002; Kuhn and Schanberg 1998; Rinaman, Banihashemi et al. 2011). Our study now identifies specific changes to protein expression in the brain that are caused by ELA in the form of MS, which may have a role in mediating neural plasticity and increasing vulnerability to drug use and addiction.

1.2 INTRODUCTION

1.2.1 Stress and its molecular effects

It has been suggested that repeated exposure to stress is capable of sensitizing dopaminergic neurons, leading to an increase in dopaminergic signaling when exposed to drug (Goeders 2003). In this aim, we examined changes in protein expression that occur as a consequence of exposure to the stress model of ELA and may underlie an increased vulnerability to drug use and dependence in adulthood. Specifically, we tested the ability of ELA in the form of MS to alter the expression of proteins associated with dopaminergic signaling and synaptic function in adult male Wistar rats. Wistar rats were chosen for this study because they are often used in addiction studies and are widely used to model various human diseases (Iannaccone and Jacob 2009; Spanagel 2017).

Stress, caused by adversity, is a systemic challenge that is capable of altering protein expression in the central nervous system through the activation of the HPA axis. Corticosterone (Cort), the principal glucocorticoid hormone that is produced by the HPA axis and regulated by hypothalamic corticotropin-releasing hormone (CRH) and anterior pituitary adrenocorticotropic hormone, is responsible for many effects of stress. The receptors for Cort and CRH are widely expressed in the brain and capable of affecting several neurological functions (Aisa, Tordera et al. 2007; Anisman, Zaharia et al. 1998; Lowery and Thiele 2010). CRH receptor antagonists, administered through an intracerebroventricular cannula, are capable of reversing the increased intake of ethanol seen during self-administration following footshock, suggesting that CRH plays a role in sensitization to drugs of abuse following stress (Liu and Weiss 2002). MS is a well-characterized method of inducing stress that can cause long-lasting changes in the activity of the

HPA axis and the expression of CRH; it also enhances the expression of arginine vasopressin (AVP) receptors in the paraventricular nucleus of the hypothalamus, which bind to AVP and further regulate HPA axis function (Anisman, Zaharia et al. 1998). ELA in the form of MS can reduce DNA or RNA synthesis, dysregulate neuroendocrine function, suppress reactions to major trophic hormones, and can even cause changes in the assembly of circuits in the brain (Card, Levitt et al. 2005; Kuhn and Schanberg 1998).

Several lines of evidence demonstrate that when a social type of adversity, such as MS, is delivered during early-life in rodents, modifications to the HPA axis can be long-lasting and impact animal behavior significantly (Aisa, Tordera et al. 2007; Anisman, Zaharia et al. 1998; Banihashemi, O'Neill et al. 2011; Card, Levitt et al. 2005; Hill, Kiss Von Soly et al. 2014; Huot, Plotsky et al. 2002; Kalinichev, Easterling et al. 2002; Kinkead and Gulemetova 2010; Kuhn and Schanberg 1998; Roceri, Hendriks et al. 2002; Romano-Lopez, Mendez-Diaz et al. 2015; Ryan, Musazzi et al. 2009). Social isolation experienced in early-life (within the same age window as MS is typically applied), for example, can increase cocaine self-administration in rats tested months later in adulthood (Kosten, Miserendino Mj Fau - Kehoe et al. 2000). This change is thought to be mediated through alterations in the activity of the HPA axis that were caused during early-life and persisted into adulthood, leading to greater dopamine release after exposure to a drug of abuse. Stress, therefore, is a highly relevant and systemic stimulus that has the ability to alter protein expression and response mechanisms in many neurological systems.

1.2.2 Specific downstream effects of stress activation

In addition to impacting gene transcription, the Cort hormone released in response to stress or adversity can also affect the brain in terms of structural plasticity and epigenetic

alterations. Cort can modify excitatory responses and plasticity in the brain through the binding and activation of mineralocorticoid and glucocorticoid receptors, and subsequently leading to an increased release of glutamate, the major endogenous ligand for the NMDAR (McEwen, Bowles et al. 2015). Activation of NMDARs can then cause synaptogenesis and plays an integral role in long-term potentiation (LTP) and long-term depression (LTD) (Luscher and Malenka 2012). LTP and LTD are respectively defined by either a long-term increase or decrease in the activity of a specific synapse, responses that are further associated with the increased recruitment/expression or internalization of synaptic receptors.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine synthesis and is thus critical for the production of dopamine. TH converts tyrosine into L-DOPA, which is then converted into dopamine by DOPA decarboxylase in the cytoplasm of dopaminergic neurons (Purves 2012). Dopamine is packaged into synaptic vesicles by a vesicular monoamine transporter (VMAT) and released into the synapse as a consequence of neuronal stimulation. Dopamine can then bind to any of five types of dopamine receptor that are expressed on the membrane of pre- or postsynaptic cells; the dopamine receptor subtypes of interest in this project are D_1 and D_2. Excess dopamine in the synaptic cleft undergoes reuptake into the presynaptic neuron via the dopamine transporter (DAT) and/or is catabolized by monoamine oxidase (MAO). Signaling through presynaptic D_2 autoreceptors inhibits TH, leading to a reduction in dopamine production. The addition of methamphetamine into this system leads to an increase in the amount of dopamine available in the synapse. Methamphetamine increases the activity of TH, leading to an increase in the production of dopamine (Volkow, Chang et al. 2001). Repeated exposure to methamphetamine leads to a redistribution of a VMAT isoform, VMAT-2, causing a

decrease in the number of vesicles available for dopamine reuptake as well as an increase in cytoplasmic dopamine, potentially resulting in neurotoxicity (Riddle, Fleckenstein et al. 2006). Methamphetamine also inhibits the catabolism of dopamine via MAO, leading to a persistent increase in synaptic dopamine levels (Kitanaka, Kitanaka et al. 2006). When methamphetamine is metabolized to amphetamine, it causes a reversal in the transport direction of DAT, causing dopamine to efflux from the presynaptic cell cytoplasm into the synapse (Riddle, Fleckenstein et al. 2006). Similarly, repeated exposure to amphetamine causes DAT to become phosphorylated and internalized by the neuron, leading to a decrease in the reuptake of synaptic dopamine (Riddle, Fleckenstein et al. 2006). Altogether, these effects of methamphetamine, summarized in **Figure 1**, amount to an increased availability of DA in the synapse. It is noteworthy to mention that the expression of these dopaminergic components is tightly linked to drug addiction and is likely affected by different stressful stimuli.

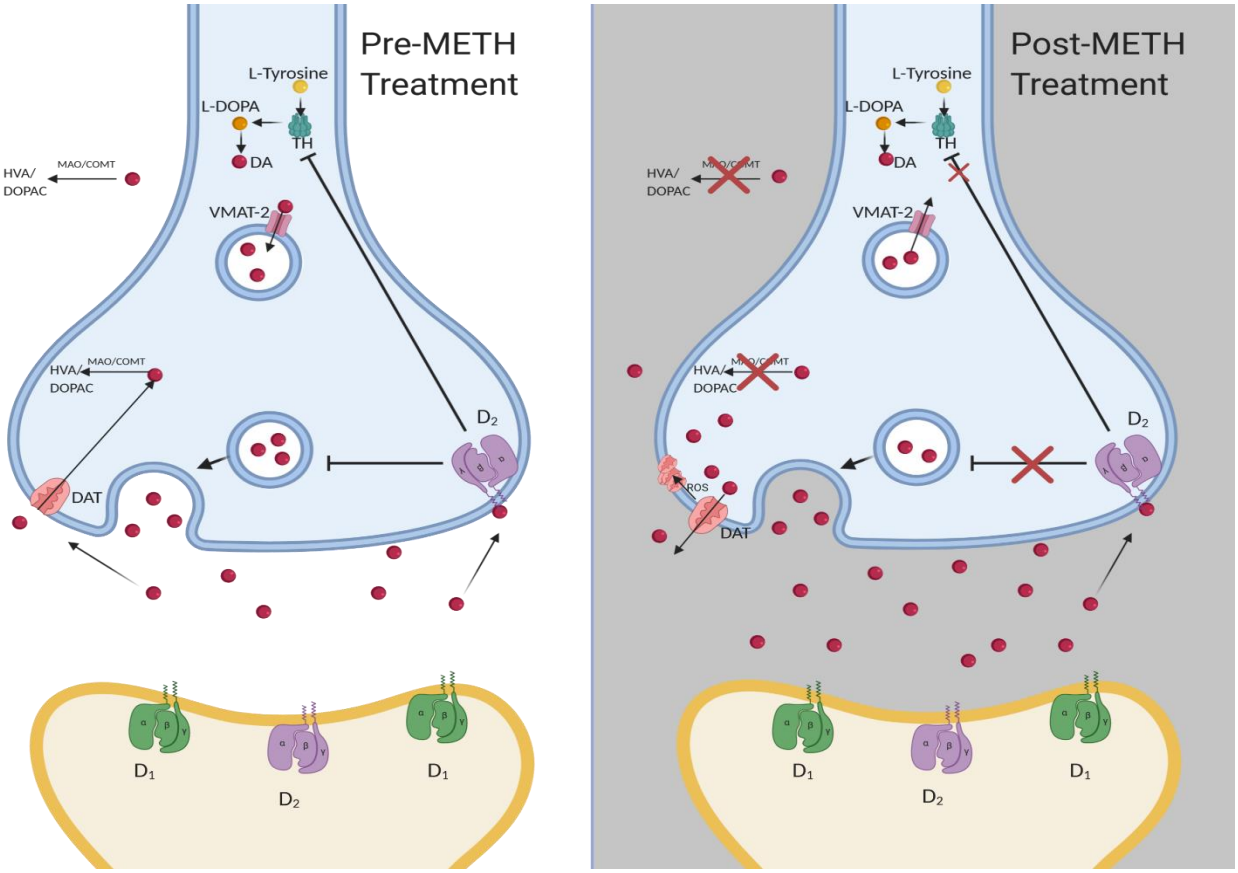


Figure 1: The effects of methamphetamine at the synapse.

This image summarizes the effects of methamphetamine on the function of various molecules within the dopaminergic synapse. Amphetamine, a metabolite of methamphetamine, reverses the transport direction of the dopamine transporter (DAT), while simultaneously reducing feedback through the D_2 auto-receptor, leading to an increase in tyrosine hydroxylase (TH) function and dopamine (DA) synthesis. It also reduces DA breakdown by monoamine oxidase inhibitor (MAO) and catechol-*O*-methyltransferase (COMT). The overall effect of methamphetamine is to increase the amount of extracellular dopamine in the synapse. D_1/D_2, dopamine receptors 1 and 2; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenyl acetic acid; VMAT-2, vesicular monoamine transporter-2.

Created with BioRender.com.

1.2.3 The reward circuitry of the brain

Dopamine plays a critical role as a neurotransmitter in the brain and is perhaps best known for its involvement in motor behavior and drug-induced motivated behavior. The complex neural circuitry underlying reward, a primary focus of this work, has been well studied and involves multiple brain regions and synaptic inputs as indicated in **Figure 2**. The NAcc, a brain region which is known to help mediate motivated behaviors, is embedded within the reward circuitry and relies on dopaminergic inputs from the ventral tegmental area (VTA), glutamatergic signals from the thalamus, HIP, basolateral amygdala, and mPFC, as well as GABAergic inputs from the VTA (Ikemoto and Panksepp 1999; Koob 1992). The NAcc also sends GABAergic signals to both the VTA and the ventral pallidum. The NAcc is divided into two morphologically and functionally distinct sub-regions: the shell and core. Cell types in both the shell and core consist of D_1- and D_2-receptor-expressing medium spiny neurons, with the neurons in the core having a higher density of dendritic spines (Beaulieu and Gainetdinov 2011). Medium spiny neurons in the NAcc are GABAergic and play a prominent role in signaling in the basal ganglia which serves various motor, learning, and other functions (Chuhma, Tanaka et al. 2011; Enoksson, Bertran-Gonzalez et al. 2012). D_1-receptor-expressing medium spiny neurons form a direct connection between the striatum and the substantia nigra pars reticulata, forming the direct striato-nigral signaling pathway which is also critical in the control and production of movement (Beaulieu and Gainetdinov 2011; Shuen, Chen et al. 2008).

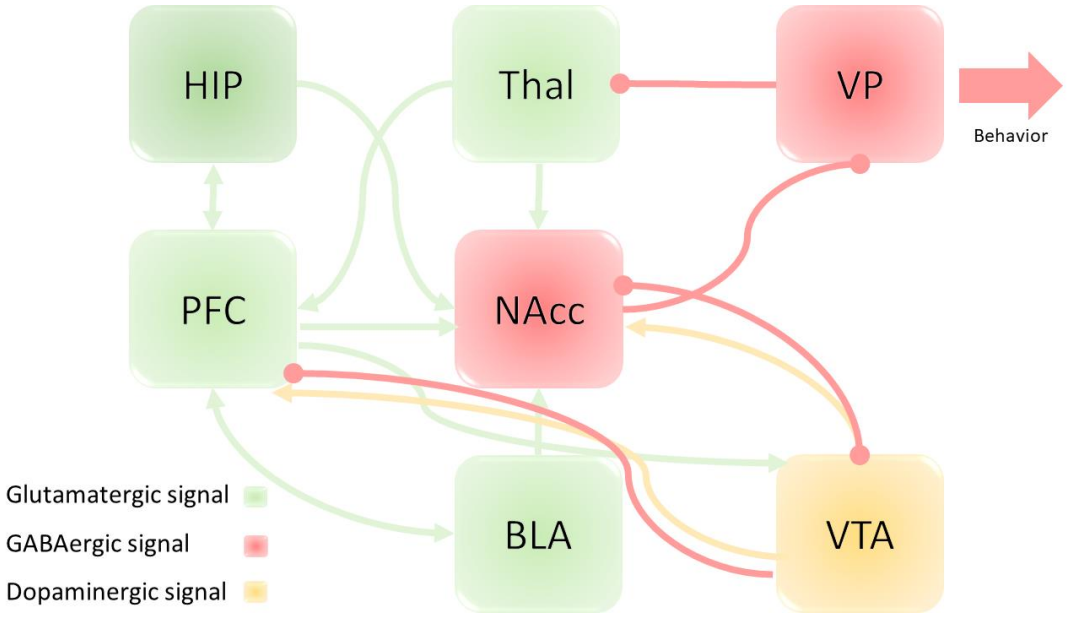


Figure 2: The reward circuitry of the brain.

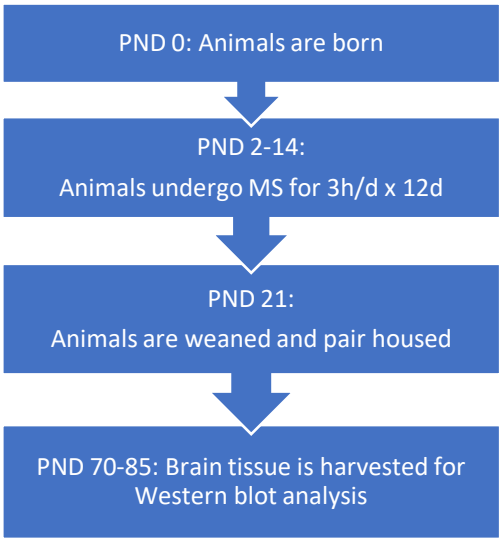
The circuitry mediating reward relies on the integration of complex excitatory and inhibitory signaling by dopamine, glutamate, and GABA. Communication among the nucleus accumbens (NAcc), ventral tegmental area (VTA), thalamus (Thal), basolateral amygdala (BLA), prefrontal cortex (PFC), hippocampus (HIP), and ventral pallidum (VP) lead to behavioral outputs that are associated with reward.

After receiving a dopaminergic signal from the substantia nigra pars compacta and the VTA, D_1-receptor-expressing medium spiny neurons send GABAergic signals to neurons of the substantia nigra pars reticulata, inhibiting their activity (Macpherson, Morita et al. 2014). D_2-receptor-expressing medium spiny neurons form an indirect pathway between the striatum and the substantia nigra pars reticulata, first by relaying through the lateral globus pallidus and the subthalamic nucleus, forming the indirect striato-pallidal signaling pathway (Beaulieu and Gainetdinov 2011; Shuen, Chen et al. 2008). After receiving a dopaminergic signal, D_2-receptor-expressing medium spiny neurons send GABAergic signals to neurons of the globus pallidus, thereby disinhibiting neurons of the subthalamic nucleus and enabling them to signal neurons of the substantia nigra pars reticulata through glutamatergic neurotransmission, increasing their activity (Macpherson, Morita et al. 2014). Together, both the direct and indirect signaling pathways allow the NAcc to communicate with the substantia nigra pars reticulata, which modulates its disinhibition (D_1) or inhibition (D_2) of the thalamus. Neurons within the shell of the NAcc are responsible for reward perception and send projections to neurons in the VTA, hypothalamus, extended amygdala, and ventral pallidum (Usuda, Tanaka et al. 1998). The core of the NAcc is responsible for the processing of motor functions related to reward and harbors neurons that send projections to the striatum, ventral pallidum, globus pallidus, and the substantia nigra (Usuda, Tanaka et al. 1998).

1.3 MATERIALS AND METHODS

Chart 1: Timetable for molecular experiments.

This flowchart depicts the experimental timeline for animal use in Section 1 of this dissertation.



1.3.1 Maternal separation

Whole litters of rat pups born in-house remained with their dams on postnatal day (PND) 0, the day of birth, and PND 1. From PND 2–14, the pups were exposed to ELA in the form of MS by physically separating them from their dams for 180 min/d between the hours of 0800 and 1100. During this process, the dam was removed from the home cage and placed in a separate cage in a vented biosafety cabinet. Pups were placed in a different cage containing surgical bedding with heat supplied via a circulating water heating pad set at 37°C and placed beneath the cage. At the end of the separation period, both the dam and the pups were returned to their original home cage and left undisturbed for the rest of the day. After the final separation period on PND 14, the litters remained continuously with their dam until PND 21, when they were

weaned and then separated by sex and pair-housed. After weaning, the male offspring were left undisturbed until adulthood (PND 60–65). Control litters were handled similarly, including during weekly cage changes, but were not separated from their dams.

Wistar rats used in these studies were bred in-house and housed (two per cage) in standard cages in a temperature- and humidity-controlled vivarium maintained on a 12:12 hour cycle with food and water available ad libitum. All animals were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (NCRC 2011), and all procedures were approved by the UTEP Institutional Animal Care and Use Committee (IACUC protocol #A-201006-1).

1.3.2 Tissue harvest

After reaching adulthood (PND 60–65), animals were lethally anesthetized (100 mg/kg pentobarbital, i.p.), and their brain tissues harvested and immediately frozen on dry ice for Western blot analyses of protein expression changes induced by MS. For these studies, the HIP, mPFC, CPu, and NAcc regions were isolated from whole brain by gross dissection and homogenized in glass hand-held homogenizers in tissue protein extraction reagent (T-PER; Thermo Scientific, Waltham, MA, USA), with protease and phosphatase inhibitors added (Thermo Scientific). Dissection of these brain regions was accomplished using surface anatomical landmarks and the rat brain atlas of Paxinos and Watson (Paxinos and Watson 1998). Coronal slices of 3 mm thickness were collected from whole brain with an acrylic brain matrix as a guide. Our specific areas of interest were further isolated using a razor blade. Samples taken from the HIP and mPFC were bilateral dissections, while samples taken from the CPu and NAcc were

unilateral dissections taken from the left hemisphere of the brain, with each sample containing the brain tissue of a single animal. Particulate matter was removed from the homogenate solutions by centrifugation and the soluble portion stored at –20°C until used for Western blot analysis.

1.3.3 Western blot analysis

BCA assay

Prior to Western blot analysis, a bicinchoninic acid (BCA) assay was performed in order to determine the total protein content of homogenates, allowing for the normalization of protein loading in Western blots. A Pierce BCA assay (Thermo Scientific) was used according to manufacturer's specifications to assess total protein content in homogenized brain samples, which were assayed in 96-well plates using bovine serum albumin (BSA) as a protein standard.

SDS-PAGE

Once the total protein content of each homogenized sample was determined, the proteins were separated by molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Twenty micrograms of total protein were mixed with 6× SDS loading buffer and phosphate buffered saline (PBS) to a final volume of 25 µL. This solution was then boiled at 85°C for 8 min and loaded onto a pre-cast Criterion® 10–20% gradient polyacrylamide gel (BioRad, Hercules, CA, USA) and run at 80 V for 20 min to stack the protein, then 140 V for 90 min to allow protein migration through the gel. Precision Plus Protein Dual Color Standards (BioRad) served as a molecular weight standard.

Western blot

Following electrophoretic separation, proteins were transferred from the gel to a polyvinylidene difluoride membrane (PVDF; BioRad) that was soaked in methanol. The membrane and gel were aligned in a transfer apparatus and transferred for 14 h at 20 V at 4°C. The membrane was subsequently washed in Tris-buffered saline with .005% Tween-20 (TBST, 10 mM; Sigma-Aldrich, St. Louis, MO, USA), and blocked using a 1% BSA/TBST (Sigma-Aldrich) solution before an overnight incubation in primary antibody solution at 4°C. Primary antibody solutions contained blocking buffer with antibodies raised against DAT, D_1 receptor, D_2 receptor, TH, PSD-95, α-synuclein, or NMDAR; with actin as a loading control. The names, concentrations and suppliers for these antibodies are found in **Table 1**. The membrane was then washed in TBST and incubated in a secondary antibody solution consisting of blocking buffer and alkaline phosphatase-conjugated antibody (either goat anti-rabbit IgG or goat anti-mouse IgG; Southern Biotech, Birmingham, AL, USA) for 1 hour at room temperature.

Densitometry

After a final TBST wash, the membrane was incubated in Immun-Star, an alkaline phosphatase substrate (BioRad) for 5 min and exposed to photographic film (Thermo Scientific, Waltham, MA, USA) for 2 min. The resulting film was then developed, scanned using a KODAK ESP 3250 model scanning bed at a resolution of 1,000 ppi, and the intensity of the signal analyzed by densitometry using LabWorks software (UVP laboratory products, Upland, CA). Alternately, some membranes were analyzed by chemiluminescence signal directly using an iBright imaging system (iBright FL1500 Imaging system, Thermo Scientific). Representative blots used for densitometric analysis are shown in **Figure 3**.

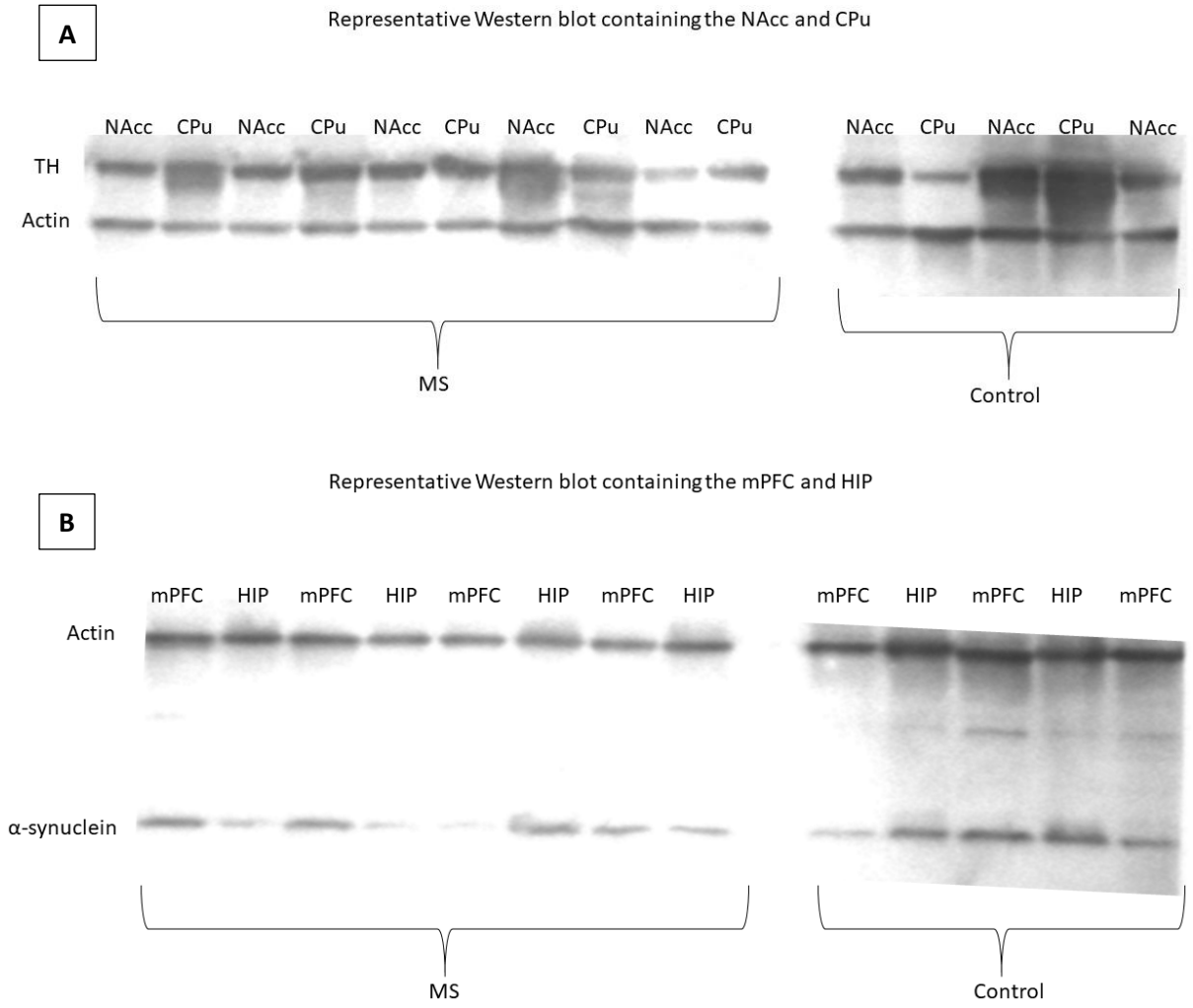


Figure 3: Representative Western blots.

Representative blots used to quantify changes in protein expression using densitometry. (A) Western blot for TH expression in the NAcc and the CPu of MS and Control animals; (B) Western blot containing α-synuclein in the mPFC and the HIP of MS and Control animals. Both blots were also analyzed for actin as a loading control. Densitometry values from Control (non-separated) rats for a given brain region and protein marker were averaged and set to 100% for subsequent comparison. The expression of individual MS samples was then presented as a percent of Control.

Statistical analysis

All Western blot experiments were performed on individual samples (n=7 or 8 per group) in triplicate and their densitometry scores averaged. Protein expression levels in samples from MS animals are presented as percent of control. Outliers, defined as having scores >1.5 standard deviations from the mean, were removed from the study. The data were analyzed for statistical significance using Student's t-test where $p \leq .05$ indicates significance.

Table 1: Antibodies for western blot.

Summary of primary and secondary antibodies used in this study including dilutions and suppliers.

Antibody	Concentration	Supplier	Catalog number
RB anti-D_1 receptor	1:1,000	Abcam, Cambridge, UK	ab81296
RB anti-D_2 receptor	1:1,000	EMD Millipore, Burlington, MA, USA	AB5084P
RB anti-DAT (H-80)	1:1,000	Santa Cruz Biotechnology, Dallas, TX, USA	sc14002
RB anti-PSD-95 (7E3)	1:1,000	Cell Signaling Technology, Danvers, MA, USA	36233p
RB anti-α-synuclein	1:1,000	Cell Signaling Technology, Danvers, MA, USA	2628s
RB anti-NMDA NR1 receptor (variant N1)	1:1,000	R&D Systems, Minneapolis, MN, USA	PPS083
MS anti-TH (clone LNC1)	1:1,000	Millipore, Burlington, MA, USA	MAB318
MS anti-Actin	1:2,000	Invitrogen, Carlsbad, CA, USA	MA5-11869
GT anti-RB IgG (H+L) (MS/HU adsorbed, affinity-purified)	1:200	Southern Biotech, Birmingham, AL, USA	4050-04
GT anti-MS IgG (H+L) (HU-adsorbed, affinity-purified)	1:200	Southern Biotech, Birmingham, AL, USA	1031-04
Abbreviations: RB, rabbit; GT, goat; MS, mouse			

1.4 RESULTS

1.4.1 Maternal separation is associated with altered expression of synaptic proteins involved in dopaminergic signaling and excitation in the brain in a region-specific manner.

In this study, the brains of Wistar rats exposed to ELA in the form of MS were subjected to tissue dissection and analysis of specific brain regions by Western blotting. Our results show that prior exposure to ELA was associated with a significant decrease ($p \leq .05$) in the expression of dopaminergic proteins in the HIP compared to levels observed in rats who were not exposed neonatally to MS (summarized in **Figure 4**). D_1 ($p=.028$) and D_2 ($p=.0038$) receptor subtype expression levels were diminished by approximately 23%, while DAT protein levels showed an apparent, non-significant ($p=.061$) decrease of more than 15% in ELA rats compared to controls. While not significant ($p=.062$), a trend toward decreased TH protein expression was also seen in the HIP. Together, the changes observed in the HIP suggest an overall decrease in dopaminergic signaling due to the decrease in its downstream receptors and the decrease in the expression of the rate-limiting enzyme for its synthesis. The non-significant decrease in the expression of DAT also suggests that while there may be a decrease in dopamine production and signaling, dopamine molecules may also be spending more time in the synapse.

Following ELA, significant changes in protein expression were observed in mPFC (summarized in **Figure 6**), including marked and significant increases in the expression of DAT ($p=.043$), D_2 receptor ($p=.034$), and α-synuclein ($p=.00016$). An apparent, though non-significant, increase in the expression of NMDA receptor ($p=.067$) was also observed in this region in association with ELA. Additionally, a significant decrease in the expression of the D_1 receptor ($p=.043$) was also detected. These data suggest a decrease in dopamine transmitter release, due

to the increase in expression of α-synuclein, a protein involved in vesicle size and regulation. The increase in D_2 receptor expression and decrease in D_1 receptor expression also suggest a disproportionate increase in signaling through the D_2 receptor, perhaps leading to inhibition of the post-synaptic neuron. While not significant, the decrease in NMDA receptor expression may also suggest a decrease in neuronal plasticity in this region.

There were several significant changes in protein expression in the NAcc following ELA, which are summarized in **Figure 8**. Specifically, a significant increase in the expression of D_2 receptor (p=.0014), NMDA receptor (p=.015), and α-synuclein (p=.0062) were observed in this brain region. Additionally, an apparent, but statistically non-significant increase in the expression of PSD-95 (p=.067) was also observed in this area. As before, these data suggest a decrease in dopamine transmitter release due to the increase in expression of the presynaptic protein α-synuclein, which is involved in vesicle size and regulation, and the increase of dopamine receptor D_2 and its scaffolding protein PSD-95. This also indicates that dopamine neurotransmitter release would likely inhibit post-synaptic neuron activation. The increase in NMDA receptor would suggest an increase in synaptic plasticity.

Following ELA, significant changes in protein expression in the CPu were observed (summarized in **Figure 10**). In this region, there were significant increases in the expression of D_2 receptor (p= .000044), NMDA receptor (p=.034), α-synuclein (p=.0012), and TH (p=.042). Furthermore, a significant decrease in the expression of DAT (p=.055) was also observed. These data suggest an increase in dopamine neurotransmitter production, due to the increase in expression of the rate-limiting enzyme in dopamine production, TH. Alternately, the increase in the expression of α-synuclein, the protein involved in vesicle size and regulation suggests a

decrease in neurotransmitter release. The increase in D_2 receptor expression, suggests that the dopaminergic signal would likely inhibit post-synaptic signaling, and the decrease in DAT suggests that dopamine neurotransmitters spend more time in the synapse, leading to an overall increase in dopaminergic signaling. The increase in NMDA receptor expression also suggests that there may be an increase in synaptic plasticity in this brain region.

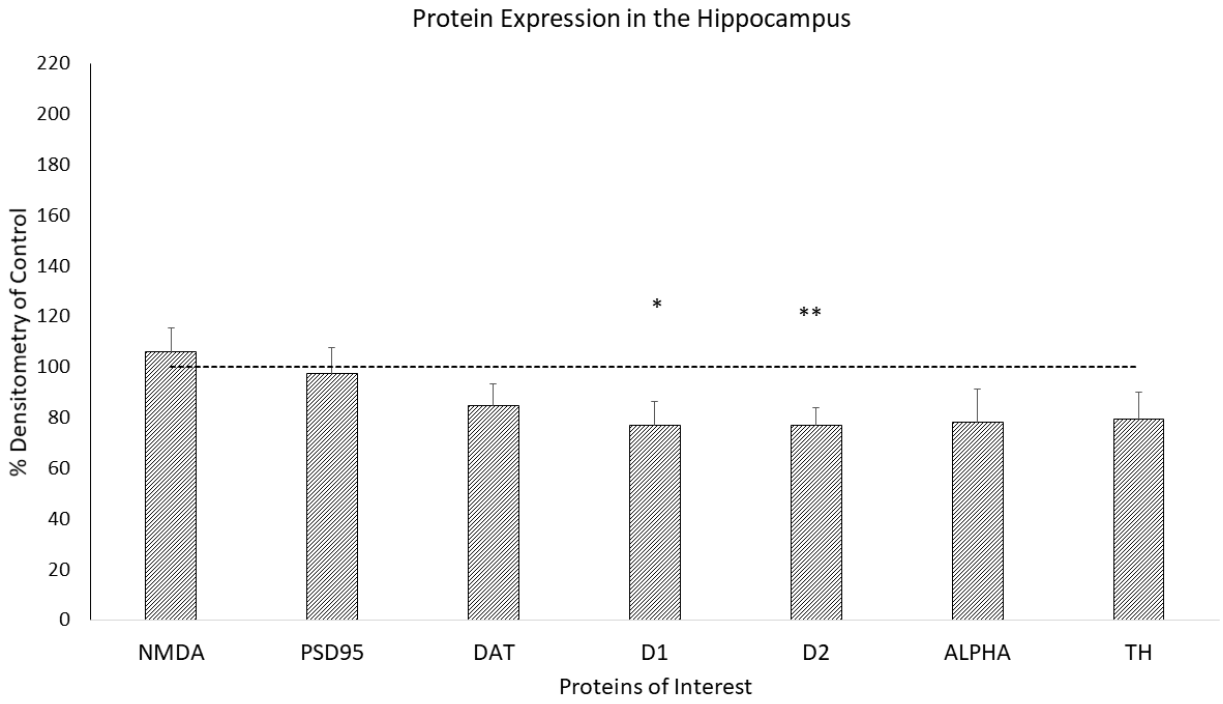


Figure 4: Protein expression in the hippocampus.

Changes in protein expression in the hippocampus expressed as percent of control (n=8). The vertical dashed line represents the expression of each marker as a percent change from controls, such that a 100% value reflects no change from control expression. * represents $p<.05$, and ** represents $p< .01$. In this study, a significant decrease in the expression of D_1 and D_2 receptor subtypes following maternal separation was observed. A trend (non-significant; $p<.07$) toward decreasing DAT and TH levels was also observed.

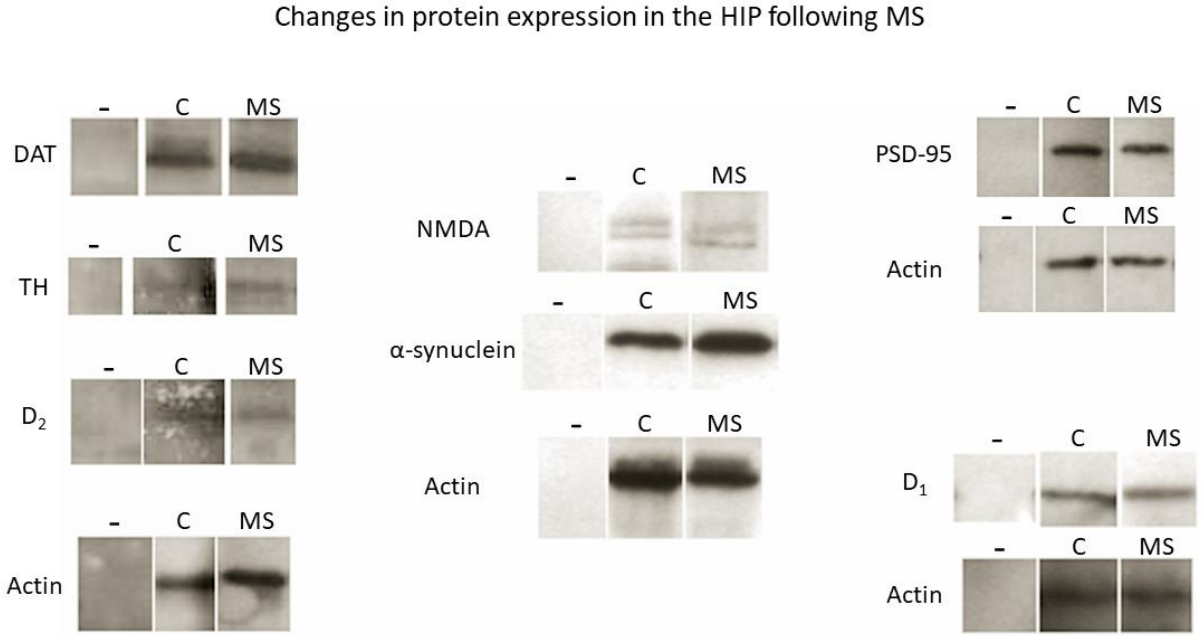


Figure 5: Representative Western blot images of proteins in the hippocampus

Representative images of bands used for Western blot analysis of D_1 receptor, D_2 receptor, DAT, NMDA receptor, PSD95, TH, α-synuclein, and actin in the hippocampus. In each panel, the negative control using no primary antibody is on the left, protein expression for the non-separated (NS) representative sample is in the center, and expression for maternally separated (MS) representative sample is on the right.

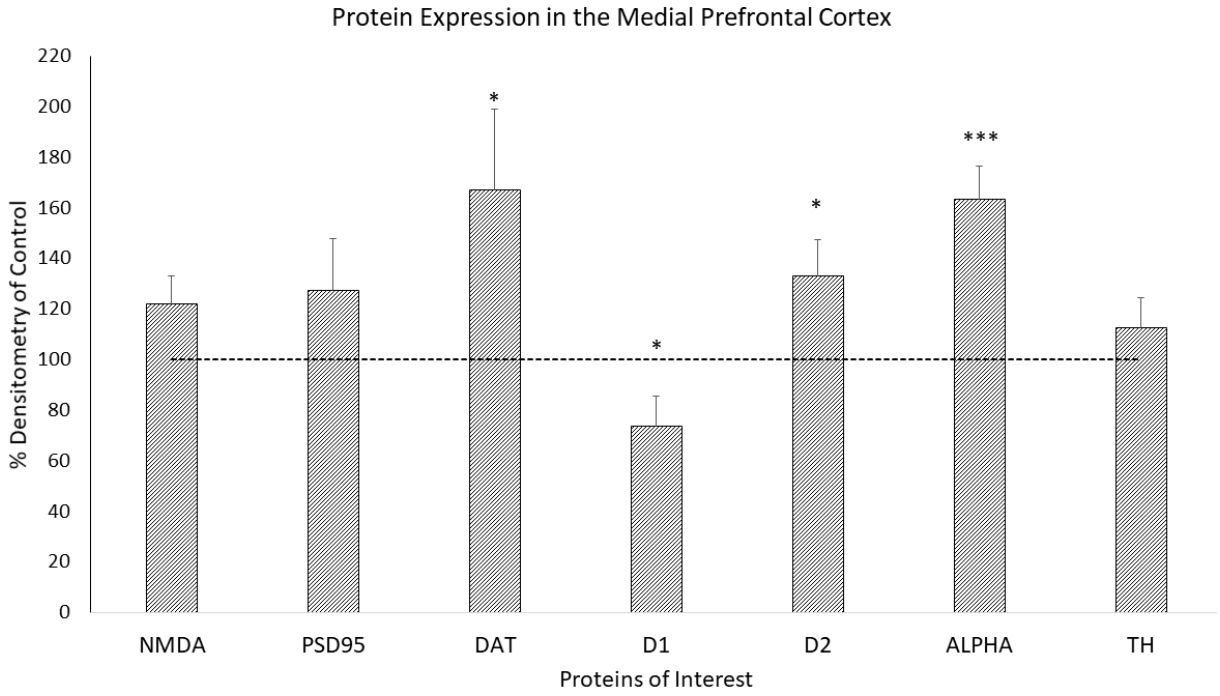


Figure 6: Protein expression in the mPFC.

Changes in protein expression in the mPFC expressed as percent of control (n=8). The vertical dashed line represents the expression of each marker as a percent change from controls, such that a 100% value reflects no change from control expression. * represents p< .05, and ** represents p< .01. In this study, a significant increase in the expression of DAT, D_2 receptor, and α-synuclein, and a significant decrease in the expression of D_1 receptor was observed following maternal separation. A trend (non-significant; p< .07) toward an increase in the expression of the NMDA receptor was also observed.

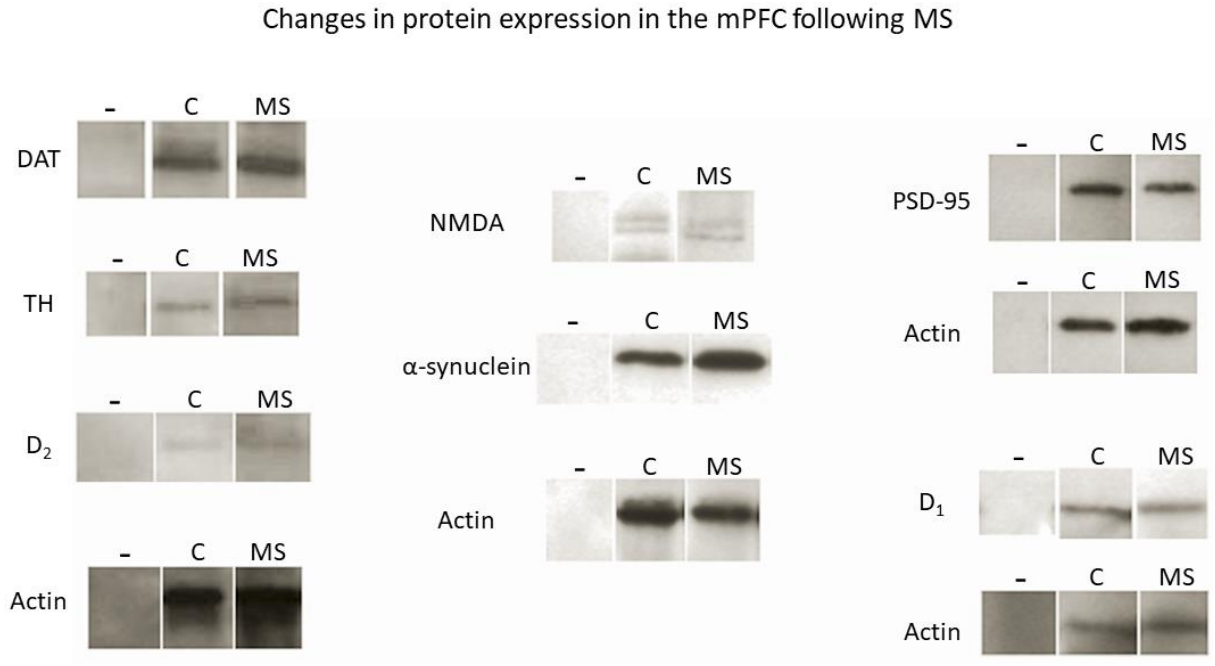


Figure 7: Representative Western blot images of proteins in the mPFC

Representative images of bands used for Western blot analysis of D_1 receptor, D_2 receptor, DAT, NMDA receptor, PSD95, TH, α-synuclein, and actin in the medial prefrontal cortex. In each figure, the negative control using no primary antibody is on the left, protein expression for the non-separated (NS) representative sample is in the center, and expression for the maternally separated (MS) representative sample is on the right.

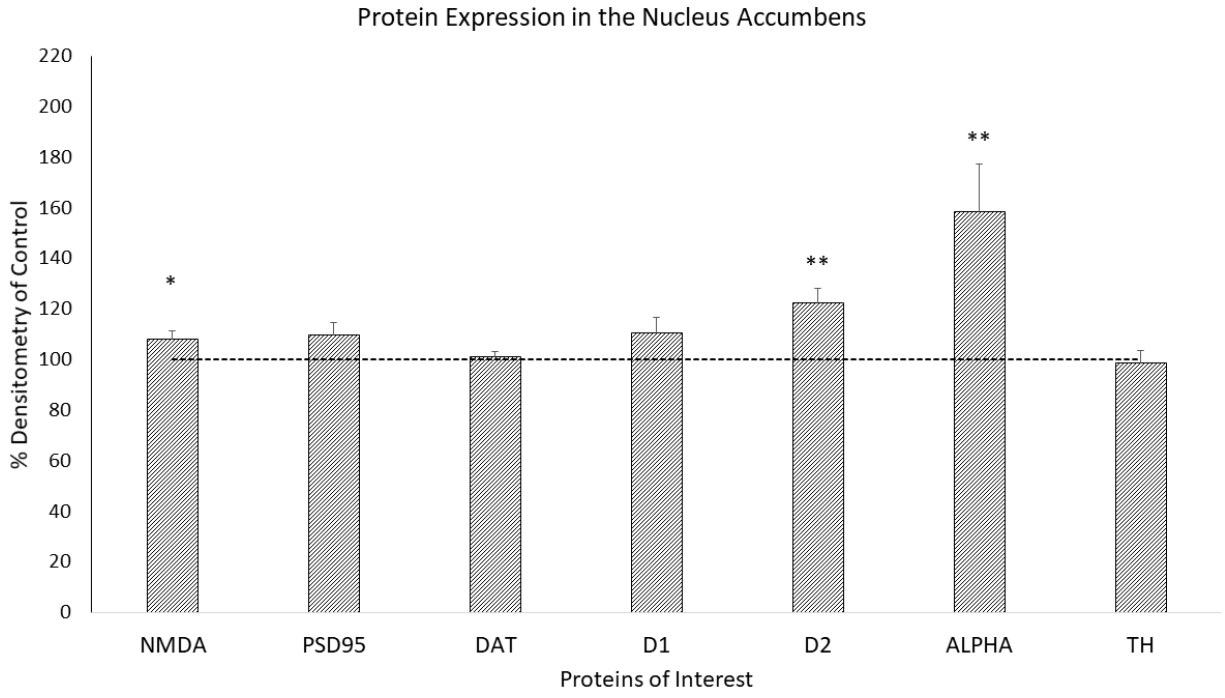


Figure 8: Protein expression in the nucleus accumbens.

Changes in protein expression in the nucleus accumbens expressed as percent of control (n=7). The vertical dashed line represents the expression of each marker as a percent change from controls, such that a 100% value reflects no change from control expression. * represents $p < .05$, ** represents $p < .01$. In this study, a significant increase in the expression of NMDAR, D_2 receptor, and α-synuclein was observed following maternal separation. A trend (non-significant; $p < .07$) toward an increase in PSD95 expression was also found.

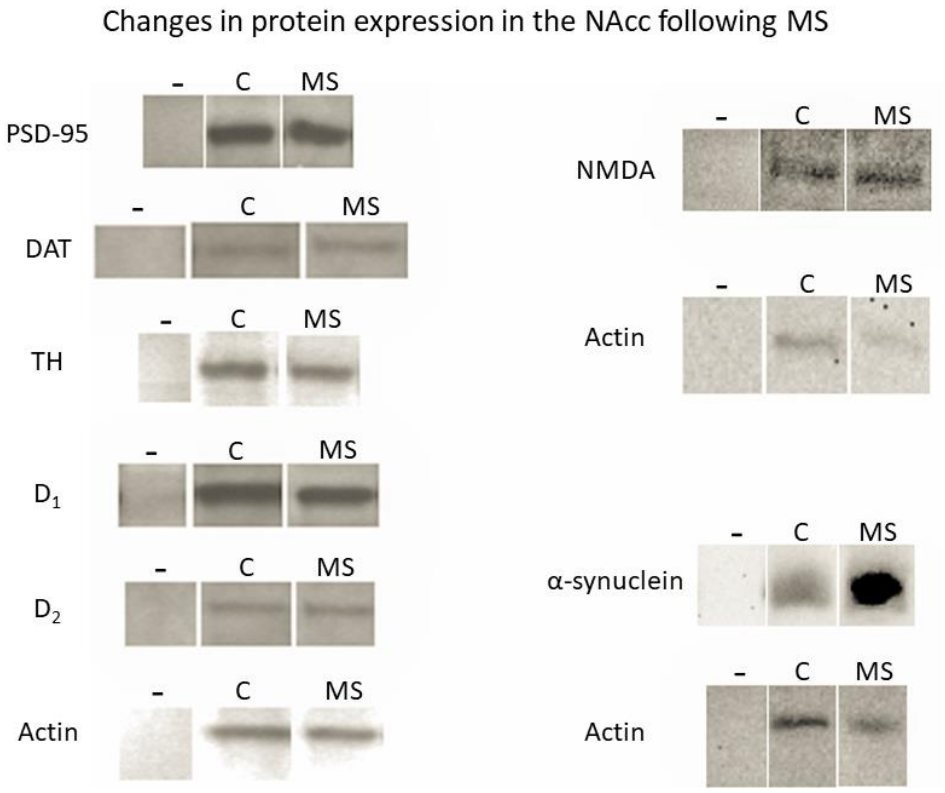


Figure 9: Representative Western blot images of proteins in the nucleus accumbens

Representative images of bands used for Western blot analysis of D_1 receptor, D_2 receptor, DAT, NMDA receptor, PSD95, TH, α-synuclein, and actin in the nucleus accumbens. In each figure, the negative control using no primary antibody is on the left, protein expression for the non-separated (NS) representative sample is in the center, and expression for the maternally separated (MS) representative sample is on the right.

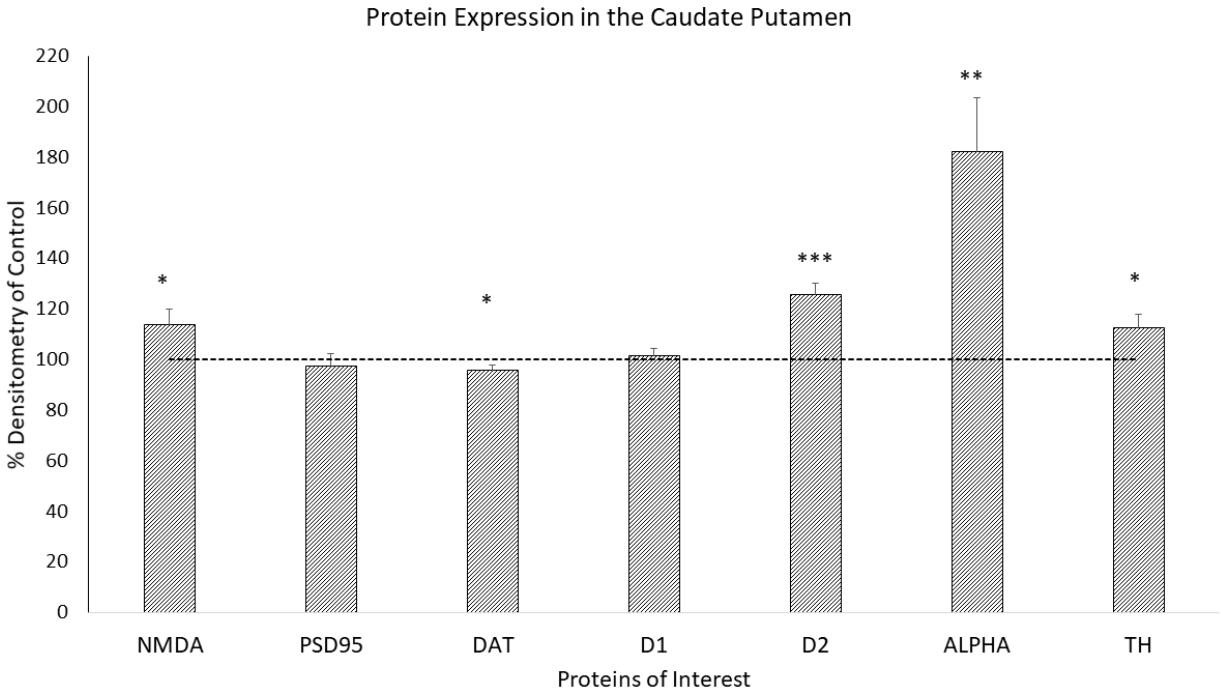


Figure 10: Protein expression in the caudate putamen.

Changes in protein expression in the caudate putamen expressed as percent of control (n=8). The vertical dashed line represents the expression of each marker as a percent change from controls, such that a 100% value reflects no change from control expression. * represents p< .05, ** represents p< .01, and *** represents p≤ .001. In this study, a significant increase in the expression of NMDAR, D_2 receptor, α-synuclein, and TH; as well as a significant decrease in the expression of DAT was observed following maternal separation.

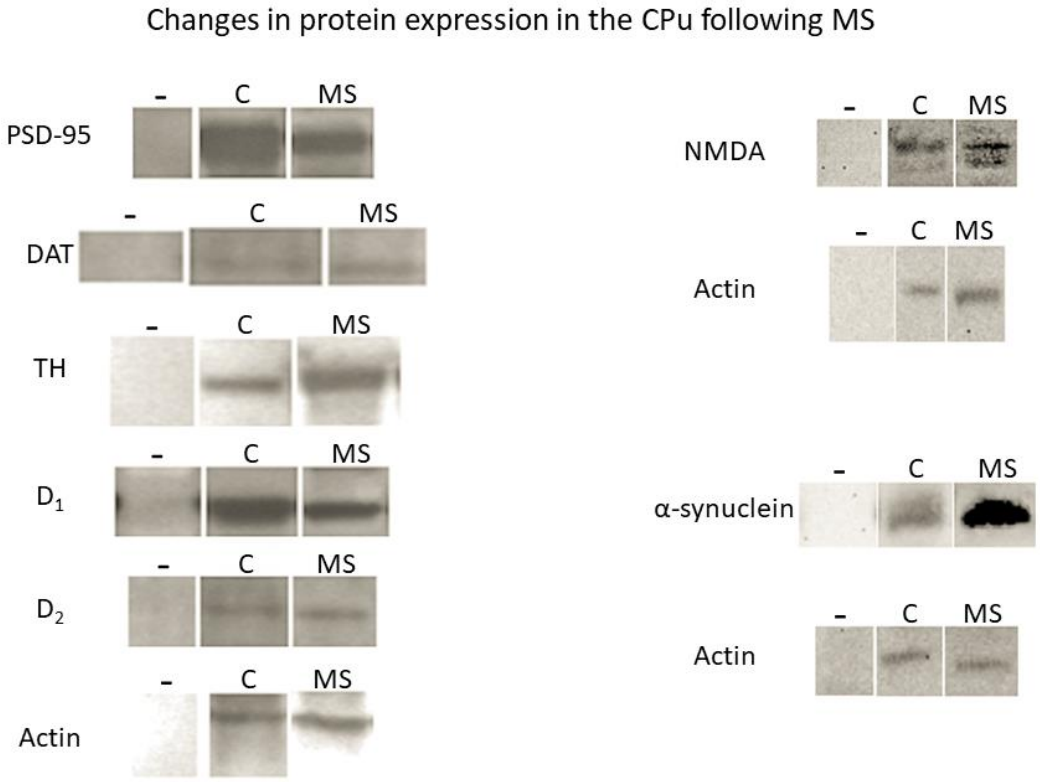


Figure 11: Representative Western blot images of proteins in the caudate putamen

Representative images of bands used for Western blot analysis of D_1 receptor, D_2 receptor, DAT, NMDA receptor, PSD95, TH, α-synuclein, and actin in the caudate putamen. In each figure, the negative control using no primary antibody is on the left, protein expression for the non-separated (NS) representative sample is in the center, and expression for the maternally separated (MS) representative sample is on the right.

1.5 DISCUSSION

1.5.1 Increased expression of dopamine receptor in the NAcc suggests an ability to decrease dopamine-mediated reward

As described above and in **Figure 2**, the NAcc plays a prominent role in the reward circuitry of the brain, integrates information from the limbic system and projects to motor regions of the brain, making the it integral for reward-mediated learning, largely through dopaminergic signaling (Day and Carelli 2007; West, Moschak et al. 2018). Increased dopaminergic signaling from the VTA to the NAcc is involved in the "binge/intoxication" stage of the addiction cycle, which is thought to differentiate so-called "casual" use of an illicit drug from addiction (Koob and Volkow 2010). An increase in dopaminergic signaling or an increase in the sensitivity to dopamine in the NAcc, would lead an individual vulnerable to addiction to a drug of abuse which, as described above and in **Figure 1**, can lead to an increase in both dopamine signaling and synthesis, at least in the rat model. Dopaminergic signaling in the brain is regulated through the precise activation and function of several regulatory proteins. When the function of these proteins is disrupted by a drug of abuse, the finely tuned balance of the system is sent into disarray. What remains unknown is whether changes can be caused by adversity prior to drug exposure and may have a synergistic or predisposing effect, leading to further disruption of dopamine signaling. Changes in dopaminergic signaling caused by stress can lead to changes in drug-taking behavior and may potentiate an individual to relapse after abstinence. In this study, we observed that exposure to adversity early in life, through the application of the stress model of maternal separation, is associated with long-lasting changes in the basal expression of proteins

which are known to also be disrupted by methamphetamine. We hypothesized that changes of this type would be discernable and may lead to an increased vulnerability to addiction.

The NAcc plays a prominent role in the normal function of the reward circuitry, and due to this role, modification of the dopaminergic signaling in this area could lead to a multitude of downstream effects which likely lead to an increased vulnerability to addiction (Koob and Bloom 1988; Koob and Volkow 2010). The increase in the expression of D_2 receptor in the NAcc seen in this study following ELA suggests a change in the function of reward circuitry. The D_2 receptor plays a complex role in dopaminergic signaling due to its role at both pre- and post-synaptic receptors (De Mei, Ramos et al. 2009). As stated above and in **Figure 1**, one function of the D_2 receptor is to serve as an autoreceptor on the presynaptic neuron working as part of a negative feedback loop which leads to the a decrease in the synthesis of dopamine. On the postsynaptic neuron, the D_2 receptor acts to inhibit the depolarization of the postsynaptic neuron by inhibiting activation mediated through cAMP by inhibiting adenylyl cyclase. The methods used in this study do not allow for the differentiation between pre- and post-synaptic D_2 receptors, but regardless of the localization of the receptor, the overall effect of the increase in D_2 receptor expression seen in this study would likely lead to a decrease in dopaminergic signaling, either via increased inhibition of the postsynaptic neuron, or the decrease in the production of dopamine neurotransmitter in the presynaptic neuron. The two main dopamine receptor subtypes, D_1 and D_2, are both implicated in the reward circuit mediated through the NAcc, and their activation has a cooperative effect on dopamine-mediated reward (Ikemoto, Glazier et al. 1997). Increased activation of D_2-receptor-expressing neurons in the NAcc has been shown to increase activity in

motivation-related tasks, which suggests that the increase in D_2 receptor expression seen in the present study may have a similar effect (Soares-Cunha, Coimbra et al. 2016).

The NAcc is also involved in the perception of other regulation of responses to stimuli that confer feelings of pleasure or aversion. Learning associated with stimuli of this type is thought to be mediated, in part, through the activation of NMDAR in the NAcc, the expression of which can be modulated by stress (Jiang, Wang et al. 2013; Kelley 2004). The increase in NMDA receptor expression seen in this study suggests that following ELA, rats may have an altered response to these stimuli. A study conducted by Núñez-Jaramillo *et al.* (2012) also documented changes in conditioned taste aversion and taste memory development that occurred as a function of NMDAR activation in the NAcc (Nunez-Jaramillo, Rangel-Hernandez et al. 2012). Specifically, the activation of NMDAR in the NAcc led to a delay in the extinction of conditioned place aversion, causing animals to retain their aversion to a stimulus for a longer time than their control counterparts. These investigators were also able to show that activation of NMDAR in the NAcc prior to a memory retrieval test interfered with the animal's ability to retrieve the memory previously acquired. Together, these data indicate an important role for NMDAR activation in the acquisition and retrieval of paired memories. Our data demonstrated an increase in the expression of NMDAR in adult rats previously exposed to ELA in the form of MS, suggesting that persistent challenges to cognitive performance may develop following this type of stress experienced long before in the neonatal period.

PSD-95 is a scaffolding protein which is responsible for scaffolding postsynaptic receptors such as D_1 and D_2 dopamine receptors, as well as NMDAR and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Due to this function, the amount of dopaminergic and

glutamatergic signaling can be enhanced or diminished depending on the presence of PSD-95, making this protein important for LTP and LTD. The increase in PSD-95 expression seen in this study indicates a need to separate the proteins PSD-95 scaffolds, the expression of which this study shows to be increased in the NAcc following ELA. Its role as a scaffolding protein allows PSD-95 to protect the brain from D_1 and NMDAR-mediated neurotoxicity that would occur in the presence of high glutamate concentrations (Zhang, Xu et al. 2009). D_1 and NMDAR subunit type 1 form an oligomer where the activation of one receptor leads to the excitation of the other in a positive feedback loop and, if left unchecked, this feedback loop can lead to excitotoxicity (Rashid, So et al. 2007; Zhang, Xu et al. 2009). An increase in the expression of PSD-95 could protect neurons from excitotoxic cell death by inhibiting the formation of D_1-NMDAR oligomers; by competitively binding to the *C*-terminus of the D_1 receptor, PSD-95 can ensure that D_1 is not available for binding to the NMDAR. One possible interpretation of our finding that MS leads to an increase in the expression of PSD-95 in the NAcc is that this may be a compensatory response that facilitates increased neuroprotection. Such an increase in protection from glutamate excitotoxicity could subsequently enhance dopaminergic signaling in the presence of a drug, such as methamphetamine, ultimately increasing the sense of reward and leading to increased use of the drug and increased probability that the user might become dependent.

The presynaptic protein, α-synuclein, plays a role in regulating vesicle size and works as a negative regulator of neurotransmission (Abeliovich, Schmitz et al. 2000; Murphy, Rueter et al. 2000). The increases in α-synuclein expression in the NAcc following ELA suggest an increase in the regulation of neurotransmitter release. Studies on α-synuclein knockout mice show that removal of this regulatory protein was associated with an increase in the release of dopamine

after paired electrical stimulation (Abeliovich, Schmitz et al. 2000). The α-synuclein protein also plays an important role in the effects of drugs of abuse (Boyer and Dreyer 2007). Animals over-expressing α-synuclein displayed a significant increase in cocaine-induced locomotion and showed an almost 100% increase in cocaine self-administration. Conversely, animals under-expressing α-synuclein, showed a decrease in locomotion and self-administration. These data suggest that the increase in α-synuclein expression seen in this study may be limiting neurotransmitter release in these animals, avoiding the depletion of dopamine in the presynaptic neuron and increasing an animal's vulnerability to drug addiction.

1.5.2 A decrease in the expression of dopaminergic proteins can lead to decreased spatial learning

The changes observed in this study for the expression of D_1, D_2, TH, and DAT following ELA suggest a total decrease in dopamine signaling in the HIP. It has recently been shown that dopamine can alter neuronal excitability and plasticity, leading to long-term potentiation (LTD) or long-term depression (LTD) in areas such as the mPFC (Kruse and Jay 2007). These effects were mediated through D_1 interactions with the NMDAR, two receptors also known to be expressed in the HIP that play a critical role in learning and memory. The D_1 and D_2 receptor subtypes in the HIP are important for spatial learning and the ability to perform certain behavioral tasks, such as conditioned place preference (CPP). More specifically, lesions to the dorsal HIP impair CPP performance in rodents (Ferbinteanu and McDonald 2001), and both the acquisition and reinstatement of CPP are dependent on the function D_1 and D_2 receptors in the CA1 region of the HIP (Assar, Mahmoudi et al. 2016). The significantly reduced expression of D_1 and D_2 in HIP samples from MS rats in the current study suggests an overall decrease in dopaminergic signaling

in this region and may lead to a subsequent decrease in the ability of animals with prior exposure to ELA to perform spatial learning tasks. We also observed a reduced level of D_1 expression in the mPFC, which could also indicate compromised plasticity in this area, as discussed below.

1.5.3 Changes in the expression of mPFC proteins may lead to decreased decision-making and conditioned learning

D_1 and D_2 receptors in the mPFC play a key role in decision-making. In this dissertation, an increase in the expression of D_2, DAT, and α-synuclein were observed as well as a decrease in the expression of D_1. These data suggest a preference for signaling through the D_2 receptor due to the increase in D_2 expression and the decrease in D_1 expression. They also suggest an overall decrease in dopamine signaling due to the increase in the expression of α-synuclein, the protein involved in vesicle size and management, and the increase in DAT expression, the protein which is responsible for the reuptake of dopamine transmitters. In a recent investigation, rats were presented with a lever-pressing task and allowed to choose between a lever that had a 100% chance of receiving a single food pellet and a lever that would deliver four pellets, but with varying levels of probability (St Onge, Abhari et al. 2011). Dopamine receptor agonists and antagonists were administered in conjunction with this task, in order to determine the roles of D_1 and D_2 in risk-based decision making. The authors showed that a blockade of D_1 receptors leads to a decrease in the probability an animal would choose the high-risk option. Administration of a D_2 receptor agonist, however, led to a breakdown of the decision-making process and animals chose the high-risk lever even when it was least likely to deliver food. When paired with a negative stimulus, D_1 receptor activation has also been shown to facilitate the formation of aversive memories (Castillo Diaz, Kramar et al. 2017). Another study was also able to show that

D_2 receptor activation leads to an increase in errors in a delayed response task while D_2 receptor antagonist infusion was able to increase accuracy in this task (Druzin, Kurzina et al. 2000). The results of our study, demonstrating decreased expression of D_1 receptor protein and increased expression of D_2 receptor protein in the mPFC in association with MS, suggest that ELA may lead to a decrease in an individual's correlative memory formation and decision-making abilities. Our increased DAT and α-synuclein expression in the mPFC further suggest an overall decrease in dopaminergic signaling, but this signaling would likely be preferentially mediated through the D_2 in either the pre- or post-synaptic membrane, ultimately tending to reduce dopamine neurotransmission or again increasing the risk for impaired decision-making, respectively.

1.5.4 Increase in dopamine signaling in the CPu suggests changes in motor function

The CPu plays a large role in motor function and motivated behavior, mediated through dopamine signaling (Pelloux and Baunez 2019). In this study, a decrease in the expression of DAT and an increase in the expression α-synuclein is observed in the CPu following MS. The changes in the expression of these two proteins may help indicate the role DAT/α-synuclein complexes play in the CPu. It is known that in the CPu, DAT/α-synuclein complexes are able to form, but there are inconsistencies in the literature as to whether these complexes increase or decrease DAT function. The arguments are that either these DAT/α-synuclein complexes aggregate and remove DAT from the synapse, decreasing reuptake of dopamine, or the complexes cause DAT to cluster on the membrane, increasing dopamine uptake and subsequently leading to dopamine-induced apoptosis (Lee, Liu et al. 2001; Oaks and Sidhu 2011; Wersinger, Prou et al. 2003; Wersinger and Sidhu 2003; Wersinger and Sidhu 2005). While the findings on the function of DAT/α-synuclein complexes are unclear, both of these hypotheses suggest that DAT/α-

synuclein complexes may change cell function by modulating DAT (Bellucci, Navarria et al. 2011). Our data show a decrease in DAT expression while also showing a strong increase in α-synuclein expression in the CPu, which may suggest enhanced neurotransmission due to the increase in synaptic dopamine resulting from the increase in release and the decrease in reuptake.

Dopamine signaling in the CPu plays a large role in motor behavior, which is mediated through D_1 and D_2 receptors via the direct and indirect pathways of signaling (Self and Nestler 1995). Together, these pathways lead to activation of the motor cortex. Activation of the direct pathway, mediated through D_1 receptor activation from the substantia nigra pars compacta, disinhibits the thalamus, leading to the activation of the motor cortex. Concomitantly, inactivation of the indirect pathway, mediated through D_2 receptor activation from the substantia nigra pars compacta, disinhibits the globus pallidus, which then disinhibits the subthalamic nucleus, leading to disinhibition of the thalamus and its activation. The increase in the expression of D_2 receptor protein found in this study, therefore, suggests an increased inhibition of the indirect pathway, leading to an increase in unwanted motor activation. The increase of dopamine function in the CPu following MS is potentially further enhanced by the increase in the expression of TH, the rate-limiting enzyme in dopamine function, in this area. In the CPu, NMDA receptors regulate the activation of dopaminergic neurons as well as the production of TH (Ravenscroft and Brotchie 2000). The upregulation in the expression of NMDA receptor in the CPu also suggests an increase in dopamine signaling in this area. Together, the increase in dopamine production and increase in dopamine signaling may have a synergistic effect, vastly increasing dopaminergic activity in this area.

A potential limitation of this study is that the brain regions were collected by gross dissection and may therefore have contained tissue from surrounding regions. Our collection of NAcc samples, for example, leaves us unable to distinguish the NAcc “core” from the “shell” in our results. This is also true for the mPFC, as we are unable to contrast protein expression in the infralimbic versus prelimbic areas and likely did not capture the entire mPFC in our samples. For the HIP, the majority of this structure was consistently captured for our analyses, including the dentate gyrus. As the NMDAR has been shown to be predominantly expressed in hippocampal pyramidal cells, it is possible that our expression levels were effectively decreased by the inclusion of surrounding tissue. We were able to demonstrate significant increases in NMDAR expression in the NAcc and CPu, with another suggested increase in mPFC. That this effect was not seen in HIP may be a function of that specific brain region but may also be a result of our tissue collection methods. An additional limitation of this study is that we cannot causally link our observed changes in protein expression directly to ELA but can only associate them with this condition. In future studies, laser capture microdissection of specific brain regions would increase sample integrity and consistency and provide more confidence in our results. Interventional studies using pharmacological agent or genetically altered animals would help establish a causal relationship between ELA and the neurochemical changes we have demonstrated.

1.6 CONCLUSIONS

In Aim 1 of this dissertation, I was able to show that ELA in the form of MS is associated with long-lasting changes in synaptic protein expression which persist into adulthood. Significant changes were seen in the expression of multiple proteins in the NAcc, HIP, mPFC, and CPu. These changes would potentially impact a wide range of downstream effects including dopamine-mediated reward, spatial learning, decision-making, and motor function. Together, these data indicate that stress, when delivered early in life, can not only induce changes in brain systems that impact reward, and even be somewhat able to mimic changes caused by drugs of abuse, but can also affect multiple diverse systems from motor function to learning and memory. These data increase our understanding of potential pathways through which stress might alter the brain in ways that predispose one to addiction even in the absence of actual drug use. Moreover, our observed changes were associated with ELA which occurred in the early neonatal period, but persisted into adulthood, and may set the stage for responsivity to drug at time periods much beyond that when the stress was experienced.

2.0 SPECIFIC AIM 2: BEHAVIOR

2.1 ABSTRACT

The objective of Aim 2 of this dissertation was to examine the behavioral effects of ELA on anxiety-like behavior and responses to a well-known drug of abuse using light-dark box and conditioned place preference paradigms. We were able to show that animals previously exposed to ELA in the form of MS exhibited an increase in anxiety-like behavior during the light-dark box test when compared to controls. This aim of the study also attempted to help address some discrepancies in the literature involving the ability of MS to increase drug use and addiction vulnerability. Few studies have been published that examine MS and methamphetamine use or addiction, and the findings of these studies have not satisfactorily explained the role MS plays in greater vulnerability to addiction. Two published investigations, one using adult and the other using adolescent animals, examined changes in CPP following MS, but were unable to demonstrate a significant change in behavior consequent to MS exposure (Faure, Stein et al. 2009; Hensleigh and Pritchard 2014). Alternately, a separate study was able to show that adult animals previously exposed to MS were able to learn intravenous self-administration (IVSA) more quickly and self-administered significantly more methamphetamine than their non-stressed counterparts (Lewis, Staudinger et al. 2013). We surmised that these conflicting findings of enhanced intake without shifts in preference for this drug under following the same initial treatment conditions may have stemmed from either: 1) heightened sensitivity to the direct reinforcing effects of methamphetamine that were not detected in the CPP studies that tested only a single dose; or 2) the voluntary nature of methamphetamine consumption in the IVSA paradigm is a critical factor. In order to test the specific hypothesis that MS induces an increased

sensitivity to methamphetamine, we performed CPP using a 0.1mg/kg/injection of methamphetamine, a sub-threshold dose which does not normally cause a change in preference, in adult rats that exposed to MS in the neonatal period. The low dose of 0.1mg/kg of methamphetamine was unable to cause a change in place preference, contrary to our hypothesis, but MS animals exposed to the light-dark box prior to CPP testing did demonstrate a significant increase in anxiety-like behavior. Together, our data indicate that neonatal exposure to ELA in the form of MS increases anxiety in adults but does not appear to alter sensitivity to methamphetamine.

2.2 INTRODUCTION

CPP is a form of classical conditioning in which animals form an association between a drug or stimulus and a physical location where the drug or stimulus is given, and is useful for identifying the motivational or aversive aspects of a stimulus (Tzschentke 1998; Tzschentke 2007). Often referred to as an "all-or-none" experiment, CPP is used to identify whether a change in preference can be observed in response to a cue, but is unable to identify the degree to which a cue is perceived as pleasurable or aversive (Reichel, Wilkinson et al. 2010). Physical and social stressors have been shown to increase CPP to amphetamine, but the ability of a stressor experienced in early-life, long before access to drug occurs, to modify responses to that drug is still largely unknown (Mathews, Mills et al. 2008; Ribeiro Do Couto, Aguilar et al. 2006).

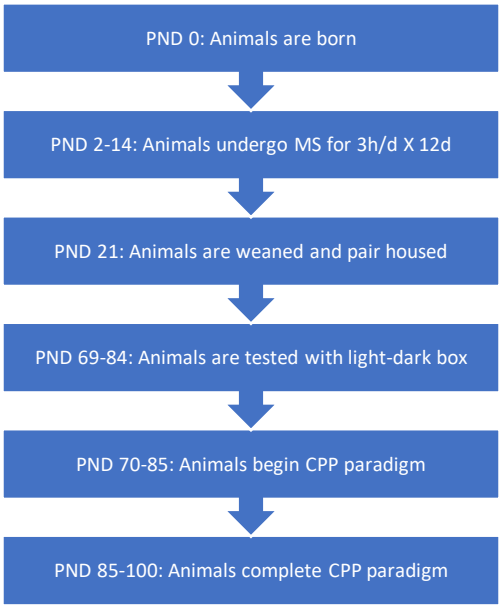
Responses to drugs of abuse such as methamphetamine can be evaluated in a CPP paradigm testing the behavior of control animals in comparison with animals exposed to stress. In the CPP model, an increased or decreased preference for the drug in question can be determined based on the amount of time an animal spends in the side of a chamber where drug was received. To date, there are few and inconsistent results in the literature regarding the effects of MS on future drug responsivity. Animals tested for CPP to methamphetamine in adolescence or adulthood after being maternally separated as neonates do not show significant changes in behavior from non-stressed controls (Faure, Stein et al. 2009; Hensleigh and Pritchard 2015). In contrast, another research group has been able to demonstrate increases in drug-taking behavior in MS rats, tested months later in an IVSA paradigm as adults (Lewis, Staudinger et al. 2013). Specifically, this study showed that MS animals escalate drug intake more quickly and consume more methamphetamine overall than controls. These findings that MS impacts drug-

taking in an IVSA scenario but does not alter the behavioral response to drug administration at a typical 1.0 mg/kg dose in a CPP setting leaves open the question of whether it is a sensitivity to methamphetamine that is increased by MS. Accordingly, in this aim, we tested this hypothesis by performing CPP on adult rats that were neonatally exposed to MS, using a lower dose of methamphetamine. If the increased drug intake shown in the IVSA study was due to enhanced drug sensitivity, we may be able to confirm this using the CPP method where increased place preference may be established with a lower dose of drug.

2.3 MATERIALS AND METHODS

Chart 2: Timetable for behavioral experiments.

This flowchart depicts the experimental timeline for animal use in Section 2 of this dissertation.



2.3.1 Conditioned place preference

CPP is a form of Pavlovian conditioning that takes advantage of the relationship an animal may form between the effects of a drug, or stimulus, and one or more environmental cues that are present in the place and time in which the drug or stimulus is administered. Prior to the initiation of the conditioning phase of the study, a pre-test is performed to identify any bias an individual animal may have for a specific side of the CPP chamber, and to determine which room should therefore be paired with the drug. During conditioning, animals are exposed to drug or saline on alternating days, for a total of eight days in this study. Once the animals are conditioned, they are again exposed to the CPP chamber environment, and the motivational effects of the

drug are determined by quantifying the time an animal spends in each side of the chamber. A graphical representation of the CPP chamber can be seen in **Figure 12**.

Animals exposed to ELA in the form of MS, as described in the first aim of this dissertation, underwent normal handling and cage changes until they reached adulthood and were then tested for CPP. Pre-testing began on PND 75, where the animals were given free access to a two-room CPP chamber with different visual, olfactory, and tactile stimuli for 15 minutes and their initial side preference recorded. After a five-day waiting period, the animals underwent conditioning for eight days, while sealed in one room of the chamber for 30 minutes following an i.p. injection of either methamphetamine in a saline vehicle or pure saline each day. Methamphetamine was paired with the initially non-preferred room and delivered at doses of either 0.1 mg/kg/d or 1.0 mg/kg/d. On the day immediately following the last day of conditioning, all animals were once again given free access to both rooms of the chamber for post-testing, and the amount of time spent in each side of the chamber recorded.

Statistical analysis

Conditioned place preference results were transformed to a difference score, as calculated by the percent time spent in the initially non-preferred chamber during the pre-test, minus time spent in same chamber during the post-test after conditioning. Differences scores and number of midline crosses were analyzed using a two-way ANOVA where $p \leq .05$ indicates significance. Tukey's post hoc analysis was used for this analysis.

Figure 12: The conditioned place preference chamber.

This figure is a representation of the conditioned place preference chamber used in this study. The chamber is comprised of two rooms with distinct characteristics including wall color, floor texture, and bedding properties.

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2.3.3 Light-dark box

The light-dark box test was used in adult rats prior to the CPP analysis described above to determine unconditioned, or baseline, levels of anxiety-like behavior in MS animals compared to controls. Each animal was placed inside the dark chamber of a light-dark box (**Figure 13**), given free access to both light and dark areas for a total of 5 min, and the percent time spent by the animal in the "light" portion of the light-dark box was assessed to determine baseline levels of anxiety-like behavior. It is important to note that behavioral responses of animals in this type of test are not fully comparable to some types of human anxiety, such as test anxiety, because the experimental animals are naturally predisposed to spend a disproportionate amount of time in the dark box (Bourin and Hascoet 2003).

Statistical analysis

The percent time spent in the light side of the light-dark box apparatus was compared between animals that were neonatally exposed to ELA in the form of MS, and non-stressed controls. Differences in this measure were calculated, and outliers of more than 1.5 standard deviations from the mean were removed from the study. Results were analyzed using Student's t-test where $p \leq .05$ indicates statistical significance.

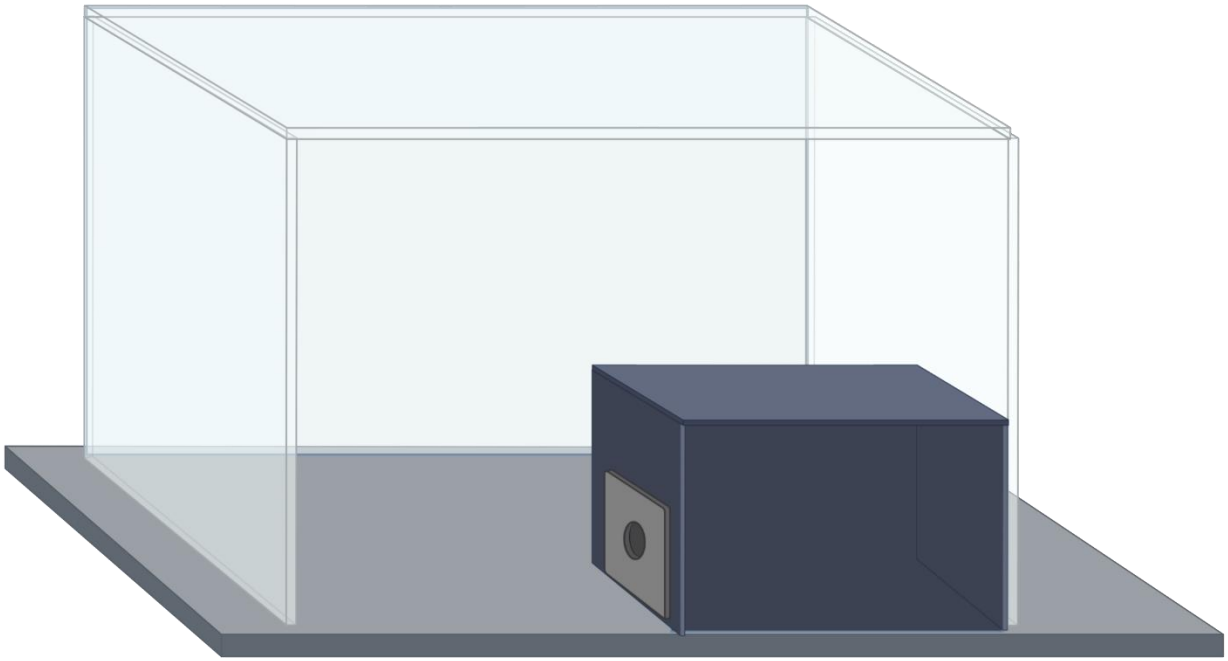

Figure 13: The light-dark box.

This apparatus contains two chambers, one opaque/black that allows limited light penetration that is nested within one that is clear and allows the entry of light.

Created with BioRender.com.

2.4 RESULTS

2.4.1 Maternal separation fails to lead to CPP with low dose of methamphetamine

We were able to demonstrate that there was a significant difference between dosage groups [F (2,34)= 7.725, p= .00172], but analysis using Tukey's post hoc analysis, these differences were shown to be strictly between animals who received 1.0 mg/kg of methamphetamine and those who received saline for both MS (p= .0183) and control (p= .0183), which is consistent with previous findings (Faure, Stein et al. 2009; Hensleigh and Pritchard 2014; Lewis, Staudinger et al. 2013), whereas there was no significant difference between animals who received 0.1mg/kg of methamphetamine and those who received saline for neither MS (p= .230) nor control (p= .9998). No significant differences were observed between MS and control groups [F (1,34) = 0.225, p= .639] and no significant interaction between dosage and ELA group were observed [F (2,34) = 1.024, p= .3698]. A summary of these findings is shown in **Figure 14** and **Figure 15**. Counter to our hypothesis, a methamphetamine dose of 0.1 mg/kg failed to shift preference in either MS or control animals. Locomotor activity was also assessed during the post-test period, recorded as the number of times an animal crossed the midline and transitioned from one chamber of the CPP apparatus to the other (**Figure 16**). No significant differences in this measure were seen between groups with either the 1.0 mg/kg or 0.1 mg/kg dose [F (2,34) = .535, p= .591], no difference was observed between MS and control animals [F (1,34) = 0.00, p= 1.0), and no interaction between dose group and ELA was observed [F (2,34) = 0.00, p= 1.0) suggesting no change in locomotor activity. Together, these data suggest that while animals who had experienced MS early in life are able to perform CPP with a standard 1.0 mg/kg dose of

methamphetamine, the neonatal ELA exposure did not lead to differences in CPP or locomotor behavior when these rats were compared to non-stressed controls.

2.4.2 Maternal separation increases anxiety-like behavior

The light-dark box data are represented as percent time spent in one chamber versus the other, and as the number of transitions that the animal makes between chambers. ELA animals showed a significant decrease in the percent time spent in the light chamber of the light-dark box apparatus, indicating an increase in anxiety-like behavior (**Figure 17A**). Additionally, there was an overall decrease in the number of times MS animals transitioned from one chamber to the next (**Figure 17B**), which also indicates an increase in anxiety-like behavior. Together, these data confirm an increase in anxiety-like behavior following MS, consistent with an earlier study in adolescents (Jin, Zhao et al. 2018).

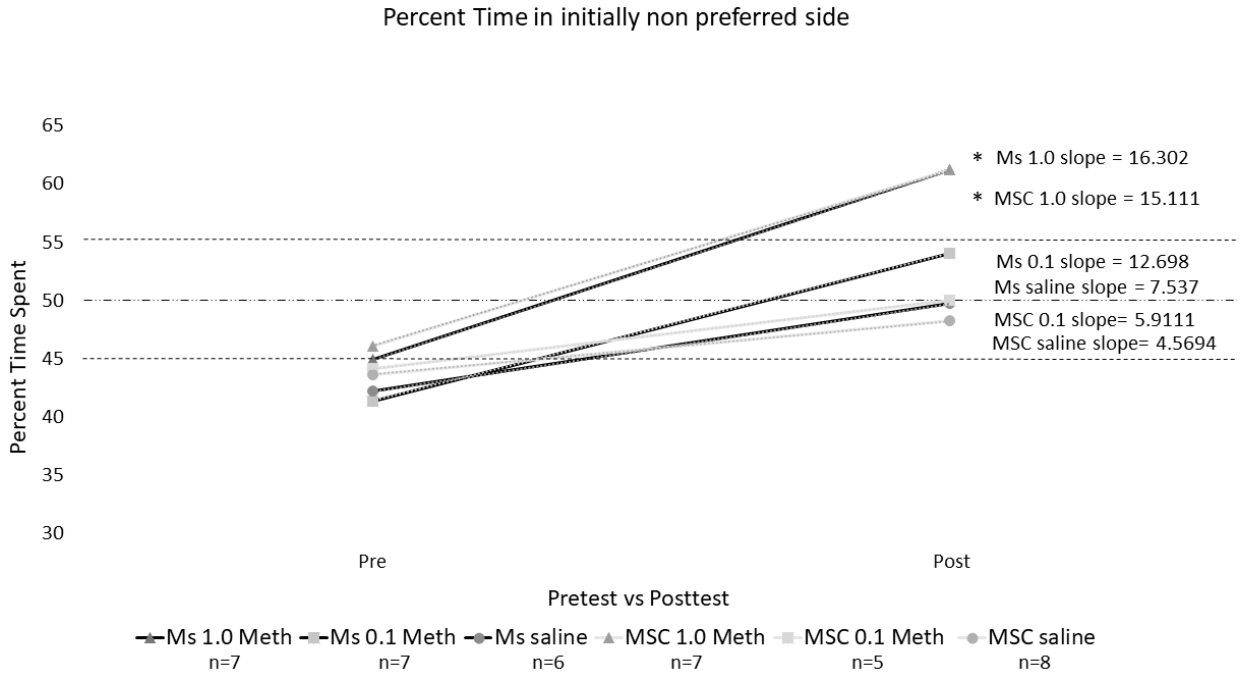

Figure 14: Average percent time spent in the initially non-preferred side.

Comparison of percent time spent in the initially non-preferred room of the conditioned place preference chamber during the pre-test and post-test, by experimental group. * indicates a significant increase ($p \leq .05$) in the difference score, suggesting an increase in place preference to the initially non-preferred chamber. The threshold for significance is seen in this graph by a transition to above the 55% line during the post-test, indicating a shift in preference.

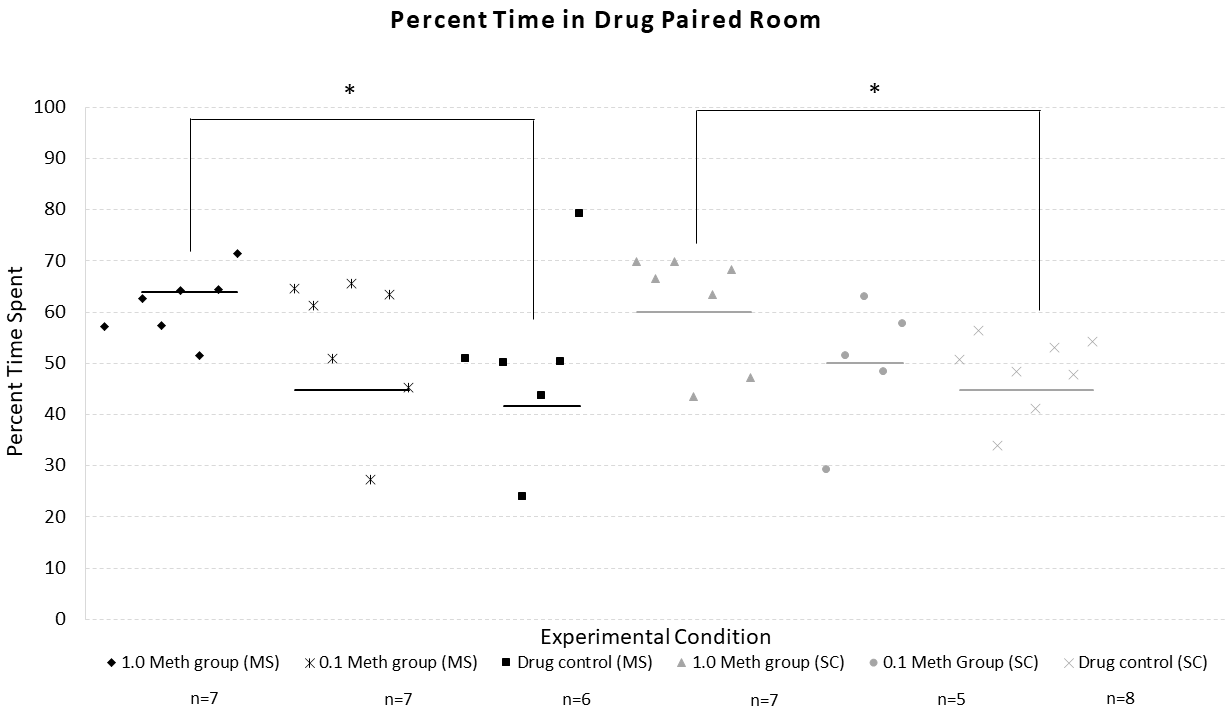

Figure 15: Individual percent time spent in the drug-paired room.

The percent time spent in the drug paired room of the conditioned place preference chamber for individuals in this study is presented here. Rats exposed neonatally to ELA in the form of MS (MS) and non-stressed controls (SC) were given either saline (drug control) or 1.0 or 0.1 mg/kg of methamphetamine, i.p. * represents a significant difference ($p \leq .05$) between the median of two groups. In this study, a significant increase is seen in the percent time spent in the drug paired room for both MS and SC groups given 1.0 mg/kg of methamphetamine.

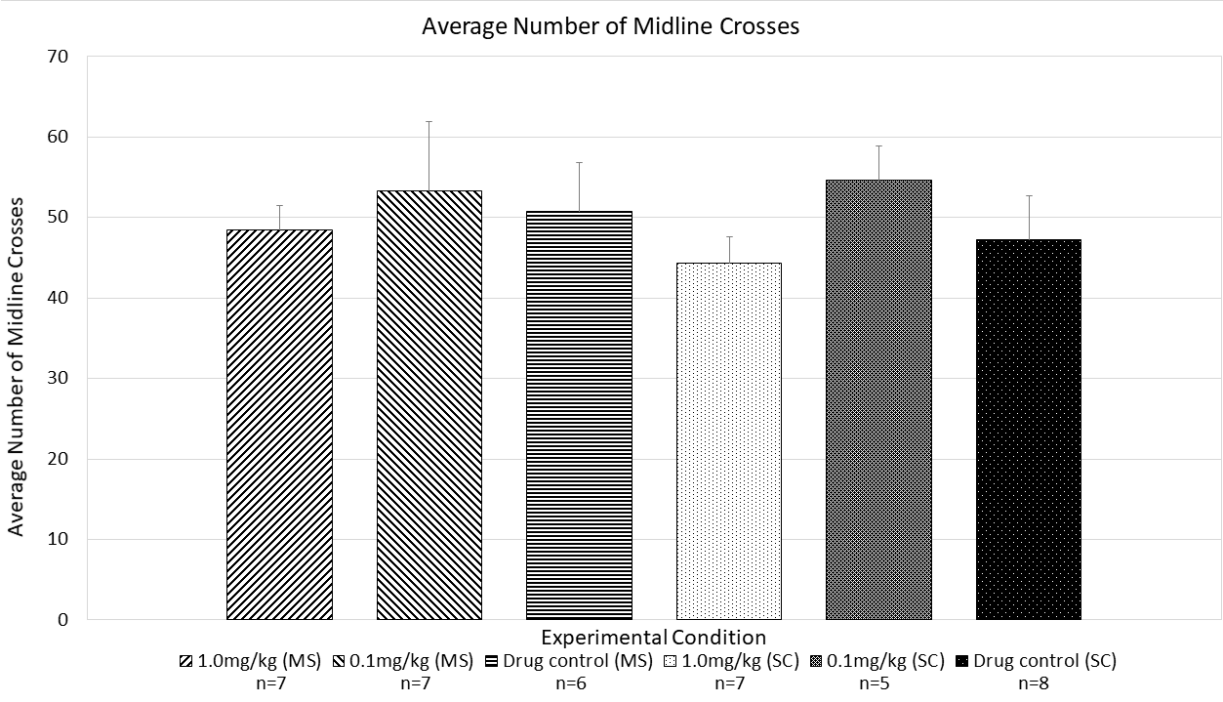

Figure 16: Locomotor behavior.

This figure shows the average number of midline crosses by the animals that occurred during CPP testing, defined as the passage of an animal's head and forepaws from one room of the chamber to the other. No significant changes were seen across treatment groups.

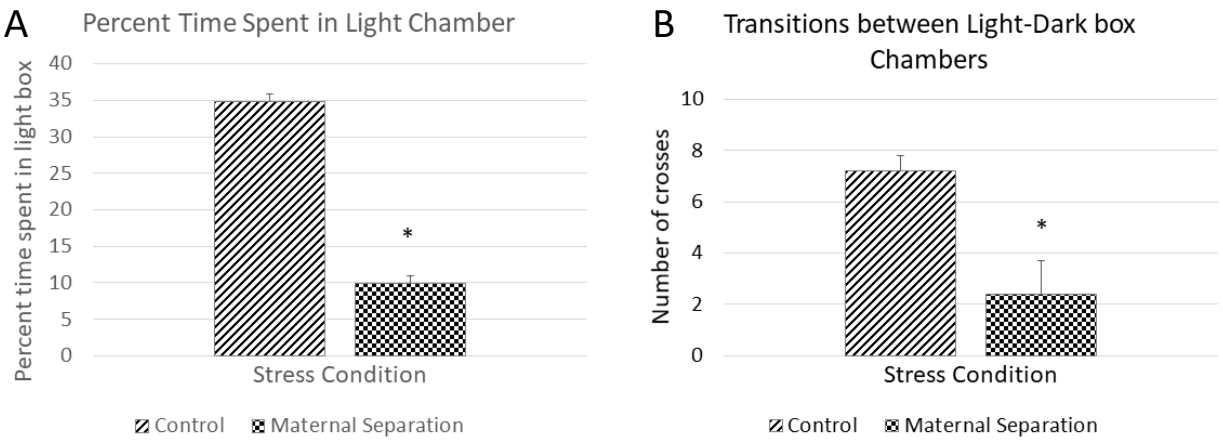

Figure 17: Anxiety-like behavior.

Comparison, by group, of the percent time spent in the light chamber of the light-dark box (A, n=5), and the number of transitions between light and dark chambers (B, n=5), between MS and control animals. * indicates a significant decrease, p≤ .05, in the observed behavior.

2.5 DISCUSSION

2.5.1 Separated animals show increased anxiety-like behavior

The light-dark box test is a simple test that is able to identify levels of anxiety-like behavior in a variety of experimental animals, including our rat model (Arrant, Schramm-Sapyta et al. 2013; Bourin and Hascoet 2003; Serchov, van Calker et al. 2016). The findings of this study demonstrate that male rats who were exposed to ELA in the neonatal period in the form of MS have an increased baseline level of anxiety in adulthood. This is consistent with findings that animals who were maternally separated have an increased level of anxiety when tested in adolescence (Jin, Zhao et al. 2018). Our data also concur with light-dark box data from adult rats tested after receiving a different type of stressor, such as foot-shock, administered during the different time of life of adolescence than those tested in this study (Lovelock and Deak 2019). Together, these findings suggest that exposure to stress can have long-lasting and significant impacts on behavior. Interestingly, the change in light-dark box activity seen adult male rats in the previously mentioned study was not seen in female rats. Thus, future work needs to replicate this work in both sexes in addition to varying age groups.

Dopaminergic signaling, through both D_1 and D_2 receptors, plays a large role in anxiety-like behavior, and disruption of this signaling is thought to lead to an increase in anxiety-like behavior (Zarrindast and Khakpai 2015). This mechanism suggests that the anxiety-like behavior observed during the light-dark box test in our study may be due to changes in dopaminergic signaling. Several studies have shown that alteration of dopaminergic signaling in several of the specific brain regions assessed in this dissertation, the HIP (Nasehi, Mafi et al. 2011; Zarrindast, Naghdi-Sedeh et al. 2010), mPFC (Lauzon, Bechard et al. 2013; Wall, Blanchard et al. 2004), and

NAcc (Zarrindast, Babapoor-Farrokhran et al. 2008) are associated with changes in anxiety-like behavior. The differential changes in the expression of the synaptic markers studied here may be involved in several aspects of the anxiety-like behavior. In Aim 1 of this dissertation, we were able to show that animals exposed to ELA in the form of MS displayed significant alterations to dopaminergic signaling molecules prior to their entry into the behavioral paradigms. These data suggest that the decreases in the expression of D_1 and D_2 seen in the HIP, the decrease in the expression of D_1 and increase in D_2 in the mPFC, and the increase in expression of D_2 in the NAcc seen in Aim 1 may play a role in the increase in anxiety-like behavior seen in Aim 2.

2.5.2 Inability to cause change in place preference suggests no change in methamphetamine sensitivity following separation

There are conflicting data in the literature where previous studies have attempted to identify changes to CPP caused by MS but were unable to show a difference between stressed and non-stressed animals (Faure, Stein et al. 2009; Hensleigh and Pritchard 2014). Data from a study using intravenous self-administration (IVSA), in contrast, indicate that MS animals escalated more quickly and consumed more methamphetamine than their non-stressed counterparts (Lewis, Staudinger et al. 2013). We believed that this discrepancy could be addressed if the all-or-none nature of CPP could allow us to demonstrate a lower threshold of response to methamphetamine and show an increased sensitivity in terms of drug preference at low doses of methamphetamine.

The findings of this study, that maternally separated animals are not able to shift preference with a low dose of methamphetamine, suggest that: 1) MS does not confer increased sensitivity to methamphetamine in adulthood; or 2) the CPP procedure itself lacks the ability to

detect MS-induced increases in sensitivity to methamphetamine at low doses. The changes in protein expression following MS that were found in Aim 1 of this study corroborate the latter idea. As described above, increases in the expression of D_2, NMDAR, and α-synuclein in the NAcc, a brain region specifically in reward-mediated learning, and increases in the expression of D_2, DAT, and α-synuclein as well as the decrease in the expression of D_1 in the mPFC, an area responsible for decision-making; suggest that these animals are susceptible to changes in dopamine signaling caused by methamphetamine. The increased availability of dopamine and increase in dopaminergic signaling seen following stress would sensitize an animal to later methamphetamine use, leading to an increase in drug-taking behavior as seen in IVSA studies (Lewis, Staudinger et al. 2013). Interestingly, MS animals demonstrated CPP to a standard (1.0 mg/kg) dose of methamphetamine, equivalent to their non-stressed counterparts, despite the reduced dopaminergic signaling in the HIP that was shown in Aim 1. Perhaps this dose provides sufficient reward to overcome a stress-induced challenge to spatial learning. Alternatively, the relative impacts of regional dopamine signaling may be an important aspect of our findings. Whether increased signaling by dopamine in the NAcc is able to compensate for simultaneously reduced dopaminergic activity in HIP, allowing for or facilitating the expression of drug-taking behaviors, could be the subject of future investigations.

2.6 CONCLUSIONS

In Aim 2 of this dissertation, it was shown that while ELA in the form of MS is not associated with changes in behavioral drug responses when analyzed by CPP during adulthood, it is associated with significant changes in anxiety-like behavior. Specifically, MS animals responded equivalently to controls in methamphetamine-induced CPP at both a typical dose of 1.0 mg/kg and a sub-threshold dose of 0.1 mg/kg. Additionally, using light-dark box testing, it was observed that in adulthood, animals who were exposed to ELA as neonates spend significantly less time exploring the "light" portion of the light-dark box, indicating an increased baseline anxiety level. The data from this aim demonstrate the long-lasting impact of stressful conditions experienced during early-life and indicate the importance of minimizing anxiogenic events during this period.

3.0 SUMMARY

In this study, certain long-lasting brain and behavioral changes associated with ELA in the form of MS, were identified. Specifically, ELA is associated with both changes in the expression of synaptic proteins associated with dopaminergic and other neurotransmitter signaling, and increases anxiety-like behavior, which persist into or are manifested in adulthood. It should be noted that no direct stress measures were taken from these animals at the time of tissue harvest or behavioral testing. Given that MS is an established stress paradigm used by numerous research groups, we anticipate that our findings result from the early-life exposure to this stressor. This interpretation is strengthened by the fact that these animals were not subjected to any other intervention after the experience of MS. We cannot rule out, however, the possibility that our observed molecular and behavioral outcomes are the result of changes that occurred as a consequence of and downstream of the stress exposure. Future studies will be needed to determine if "stress" and "adversity" in early-life are the direct causes of the observed results. Alternately, we showed that MS did not cause changes in CPP behavior. The relationships between stress and addiction are complex and difficult to dissect, and in the context of this study, CPP may not be sufficiently sensitive to detect long-term effects of stress on drug taking behavior. This study was, however, able to help us understand some of the complexities of stress or early-life adversity that might be causally linked to changes in the brain and that may predispose an individual to developing drug addiction. Through our findings on the broad effects of ELA on the expression of specific markers in different brain regions, this study helps us further identify and isolate mechanisms (i.e., dopaminergic) that may lead to an increase in anxiety-like behavior. Further studies will include an examination of the markers and brain regions from this

investigation in MS animals following exposure to methamphetamine. In the future, we would also like to include females to identify any sex differences in the changes we have observed; the comparison between male and female animals is an area of study that is still too often overlooked.

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VITA

Jameel Nasser Hamdan attended The University of Texas at El Paso (UTEP), from which institution he graduated with a Bachelor of Science in Microbiology in 2013. He was a member of the NSF-STEM scholarship for his first two years at this university. During his first year, he received his first experience in research when he joined the "Science Education Alliance - Phage Hunters Advancing Genomics and Evolutionary Science" lab. Here, in 2010, he learned how to isolate, purify, and annotate the genome of mycobacteriophage, assisting in the genomic annotation of the mycobacteriophage "Airmid". Upon leaving the "Phage Hunters" lab in 2011, he joined the lab of Dr. Kristin Gosselink, where he learned several techniques used in neuroscience, including immunohistochemistry, western blot analysis, animal handling, and tissue harvest. During his time as an undergraduate in the lab of Dr. Gosselink, he traveled to and presented posters at the Experimental Biology conference in 2012 and 2013. After his graduation in 2013, he continued working with Dr. Gosselink in her lab as a Ph.D. student where he joined the Vulnerability Issues in Drug Abuse (VIDA) project. During his time as a Ph.D. student in this lab, he presented at the VIDA poster session in 2014 as well as the Society for Neuroscience poster sessions from 2014 to the present and remains a member of the Society for Neuroscience. While contemplating the study contained in this dissertation, he helped create a new protocol at UTEP for the maternal separation seen in this study.

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