EARLY-LIFE STRESS COMBINED WITH A WESTERN-STYLE DIET LEADS TO BEHAVIORAL, PHYSIOLOGICAL, AND NEUROBIOLOGICAL DYSREGULATION

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2021

Major Subject: Neuroscience

ABSTRACT

Metabolic-Mood Syndrome (MMS) is used to describe the comorbidity between multifactorial diet-related diseases (e.g., metabolic syndrome, cardiovascular disease) and mood disorders (e.g., major depressive disorder, anxiety). Although the existence of this phenomenon is now widely recognized by clinicians, the mechanisms underlying their co-occurrence are unclear. Western-style diets (WSD), which are high in both fats and carbohydrates, are known to induce physiological changes that result in dysregulated metabolic signaling (e.g., insulin, corticosterone) as well as central and peripheral inflammation. Chronic stress, which often precipitates depressive-like symptoms and other mood disorders, results in similar deficits and, not surprisingly, both WSD and stress bidirectionally exacerbate behavioral and physiological symptoms. A dramatic increase in the prevalence of major depression and diet-related disorders in adolescents has been observed over several decades, yet the mechanisms underlying this comorbidity have only recently begun to be studied. Given that adolescence is a developmental period highlighted by vulnerability to both stress and poor diet consumption, understanding the mechanism(s) underlying the combined negative effects of WSDs and stress on mood and reward regulation is critical.

To this end, various permutations of diet and stress exposure paradigms were utilized to characterize physiological and behavioral alterations. Chronic and vicarious social defeat stress were utilized during adolescence to dissect whether stress could precipitate changes in response to diets that were either high in fats and carbohydrates

(HFD) or only high in carbohydrates (LFD). The use of various stress paradigms allows for distinction between physical and non-physical stressors, whereas the use of multiple diets allows for distinction between the effects of diets high in both fat and carbohydrates and those only of high carbohydrates. Two control diets were utilized to ensure that observed effects were not micronutrient dependent. Overall, data demonstrated that the tautological relationship between metabolic and mood disorders is likely associated with obesity, behavioral abnormalities, intracellular signaling changes within mood and reward- regulation brain substrates, increased levels of pro-inflammatory cytokines, microglial involvement, and alterations in microbiome diversity.

DEDICATION

I dedicate this work to my Mom, Dad and my best friend Nadia.

And to my smolecules, Tamara and Luna Sial.

ACKNOWLEDGEMENTS

I would like to first thank my mentor Dr. Carlos Bolaños-Guzmán for his continuing support and guidance. He taught me just as much about life as he did research. He was always there to motivate me and make me feel capable when I did not.

I would like to thank my parents – Nyla and Khalid Sial, for their constant support and love, despite not knowing what exactly I have been doing over the years. My sister and best friend, Nadia; for not only helping with my ideas and writing, but for all the emotional support and comical relief over the years. Thank you for sharing all the important moments with me.

My other family − Eric and Lyonna Parise. I would not be where I am today without them. They have contributed more to my growth and development than they will ever know. They helped me find the port in the storm.

My colleagues at Texas A&M University and Mt. Sinai Icahn School of Medicine. I am forever grateful to Dr. Eric Nestler and his lab for their invaluable help and for welcoming me into their family.

Finally, I thank my wife and partner in crime − Tata. Thank you for your patience support and love. None of it would be possible without you.

CONTRIBUTORS AND FUNDING SOURCES

This work was supervised by a dissertation committee consisting of Dr. Carlos Bolaños- Guzmán of the Institute of Neuroscience and Department of Psychological and Brain Sciences [Advisor] and Dr. Heather Lench of the Department of Psychological and Brain Sciences9 [Outside Department] and Dr. Justin Moscarello of the Institute of Neuroscience and Department of Psychological and Brain Sciences [Home Department] and Dr. Linglin Xie of the Department of Nutrition [Outside Department].

The microbiome 16S sequencing data analyzed for Chapter IV was conducted by LC Sciences and further interpretation was provided by Dr. Drew Kiraly and his laboratory at Mt. Sinai Icahn School of Medicine in the Nash Family Department of Neuroscience.

Assistance in magnetic sorting protocol for microglia in Chapter V was provided by Dr. Leanne Holt. Quantification of the RNAscope images in Chapter V was perform in ImageJ with program designed by Arthur Godino and Dr. Marine Salery.

Graphical work shown in Appendix B was created using BioRender. All other work conducted for the dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by a scholarship from Stuart Moldaw Foundation. This work was also made possible in part by NIDA under Grant Number [R01DA046794]. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIDA.

NOMENCLATURE

AAV	Adeno-associated virus
ANOVA	Analysis of variance
BBB	Blood brain barrier
BMI	Body mass index
C57	C57BL/6J
CD	Control Diet
LFD	Low fat/ high carbohydrate diet
CNS	Central nervous system
CON	Control
CORT	Corticosterone
CPP	Conditioned place preference
CSDS	Chronic social defeat stress
CVD	Cardiovascular disease
DA	Dopamine
DAPI	4′,6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
ES	Emotional/psychological stress
FDA	Food and Drug Administration
FLX	Fluoxetine
HDL	High-density lipoprotein

HFD	High fat/high carbohydrate diet
HIP	Hippocampus
IL	Interleukin
IR	Interaction ratio
IRS	Insulin receptor substrate
MDD	Major Depressive Disorder
MetS	Metabolic Syndrome
MOR	Morphine
MSN	Medium spiny neuron
NAc	Nucleus Accumbens
NC	Normal chow
PCR	Polymerase chain reaction
PD	Postnatal day
PS	Physical stress
PTSD	Post-traumatic stress disorder
SAL	Saline
SIT	Social interaction test
TRD	Treatment resistant depression
VSDS	Vicarious social defeat stress
VTA	Ventral Tegmental Area
WSD	Western-style diet

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The co-occurrence of mood disorders (e.g., major depressive disorder, generalized anxiety disorder, bipolar disorder, etc.) with diet-related diseases (e.g., metabolic syndrome, cardiovascular disease, etc.) has become a well-recognized phenomenon in recent years despite the underlying mechanisms being severely understudied (Pan et al., 2012; Tang et al., 2017). This paucity of research is likely due to the behavioral and biological complexities of these individual disorders and the involvement of multiple biological systems, including neurobiological, immunological, and microbiological (Milaneschi et al., 2019). This comorbidity, coined the metabolic-mood syndrome, has recently become popular in both clinical and preclinical research (Mansur et al., 2015). Studies often employ diet-induced models of metabolic dysfunction and separately use stress paradigms to induce depressive-like symptoms (Krishnan and Nestler, 2011; Speakman, 2019). This experimental approach has likely generated the lack in translational research models that aim to understand how stress and diet interact simultaneously to precipitate symptoms like those present in various comorbid mood- and diet-related disorders. Adolescence is a period of enhanced vulnerability to stress, which can precipitate the emergence of major depressive disorder (MDD) and anxiety-related disorders (Eiland and Romeo, 2013), often causing life-long detriments (Kessler et al., 2007; Merikangas et al., 2010). MDD is highly comorbid with obesity and, more broadly, metabolic syndrome (MetS) (van Reedt Dortland et al., 2010; Vogelzangs et al., 2014). Afflicted youth often adopt poor eating habits (Muñoz et al., 1997; O'Neil et al., 2014; Peeters, 2018), deriving sources of energy from foods high in fats and sugar (Reedy and Krebs-Smith, 2010). This is concerning as there is a bidirectional relationship between

MDD and MetS, with each predicting the onset of the other (van Reedt Dortland et al., 2010; Vogelzangs et al., 2014). The symptomatology of mood- and diet-related disorders often overlap and, though well-recognized, the mechanism(s) underlying this comorbidity is not well-understood (Mansur et al., 2015). Given the increased prevalence of both MDD (Mojtabai et al., 2016) MetS (Weiss et al., 2004) in adolescents that can carry over the years, it is crucial to address this issue early in life to harbor better coping strategies and avoid maladaptive behaviors/habits in these individuals as they progress into adulthood.

Mood disorders, such as MDD, are among the leading causes of disability worldwide (Friedrich, 2017). Among those with mental disorders, the rate of mortality is increased ~2.22-fold when compared with the general population (Walker et al., 2015). This higher mortality rate suggests a shortened lifespan of nearly 20 years affecting nearly 20% of the world's population (Manji et al., 2001). To make matters worse, the etiology of depression can vary greatly from environmental factors (e.g., stress and diet) to genetic predisposition playing critical roles (National Research Council (US) and Institute of Medicine (US) Committee on Depression, Parenting Practices, and the Healthy Development of Children, 2009). Additionally MDD can present with a wide range of symptomologies, such as increased appetite and sleep in atypical depression (Davidson et al., 1982). Subsequently utilization of the proper treatment regimen proves to be a complex, multifaceted issue, as most antidepressants used currently target monoaminergic systems and often times are not as efficacious, if at all (Krishnan and Nestler, 2008). Furthermore, refractory or treatment-resistant depression is a serious and growing concern in the younger population (Botteron and Geller, 1997; Maalouf et al., 2011) and considering that depression is also comorbid with several other disorders such as anxiety, pain, and diet-related

disorders, it severely complicates treatment options (Gul and Bali, 2017). Diet-related disorders such as cardiovascular disease (CVD) and MetS significantly influence the prognosis of MDD as it accounts for a 4-fold increase in premature death in depressed patients (Marazziti et al., 2014).

Highly prevalent and debilitating, MetS is an ailment characterized by the presence of three out of five conditions: elevated blood glucose levels, increased triglycerides, low high-density lipoprotein (HDL) cholesterol, hypertension, and abdominal obesity (Huang, 2009). The increased occurrence of MetS has drawn serious concern as it is a well-known contributing factor in diseases such as CVD, type-2 diabetes, and stroke (Gonzalez-Chávez et al., 2018). Patients with depression are five times more likely to have a cardiac event in their lifetime (Thombs et al., 2006) and the link between MDD and CVD is thought to be mediated through MetS (Vogelzangs et al., 2011). This is further complicated by the seemingly bidirectional relationship between MDD and MetS, with each predicting the onset of the other (van Reedt Dortland et al., 2010; Vogelzangs et al., 2012). MetS has been observed with a higher incidence in individuals with MDD, with those diagnosed with mental disorders being 1.58 times more likely to also be diagnosed with MetS (Vancampfort et al., 2012). Conversely, MetS has also been associated with an increased prevalence of depression but not anxiety in both men and women (Skilton et al., 2007). Several meta-analyses have postulated that weight gain and obesity link these disorders, further complicating the presentation of mood disorders (Milaneschi et al., 2019). This phenomenon is often referred to as metabolic-mood syndrome (MMS) (Mansur et al., 2015).

It has become increasingly evident that though this comorbidity is highly pervasive in the adult population, the problem often arises during periods prior to adulthood, where adolescents are highly sensitive to stress (Eiland and Romeo, 2013) and often develop unhealthy dietary habits

(Jacka et al., 2011). The first onset of mental disorder or substance abuse often occurs during childhood or early adulthood (Kessler et al., 2007), and it has been proposed that consumption of poor-quality diet is used as self-medication in response to internalization of mood disorders (O'Neil et al., 2014). Given that poor diet choices are the greatest modifiable risk factor for MetS and CVD (Peeters, 2018), most basic studies have utilized diet-induced obesity models to understand the underlying neurobiological mechanisms of MetS (Speakman, 2019). Behind nicotine smoking, obesity is the leading preventable disease worldwide (Johnson et al., 2014). Diets such as those high in fats and/or carbohydrates (i.e., western-style or pattern diet; WSD) have been studied in the context of this comorbidity. Chronic consumption of calorically dense diets has been shown to lead to dysregulation of lipids (e.g., cholesterol, triglycerides) (Asadi et al., 2020; Mensink and Organization, 2016), changes in serum hormone levels such as insulin (Esmaillzadeh et al., 2007), leptin, biomarkers for CVD (Fung et al., 2001), cognitive impairments (Francis and Stevenson, 2013; McLean et al., 2018), and development of depressive-like phenotypes (Li et al., 2017). It is integral that prevention strategies be taken early in life, to prevent improper behavior and biochemical programming and consequent long-term detriments to quality of life and functional capacity.

There is a wide range of metabolic abnormalities that are characteristic of both chronic WSD and stress exposure during early-life, with perhaps the most problematic being the manifestation of obesity, or excess accumulation of adipose tissue. Various chronic stress paradigms have resulted in dysregulation of hormones like cortisol (Pryce et al., 2002) and insulin (Rostamkhani et al., 2012), changes in reward sensitivity (Chuang et al., 2011; Warren et al., 2013) and alterations in physiological measures such as adiposity (Lin et al., 2015). Due to the paucity

of research modeling chronic stress concurrently with poor diet consumption, inferences on the overlapping mechanisms must be made from separate studies. The involvement of multiple systems have been implicated in the potentiation of mood and metabolic symptoms, such as dysregulation of immune responses (Christ et al., 2019; Wu et al., 2018) and disruption of the gut-microbiome (Foster and McVey Neufeld, 2013; Magnusson et al., 2015; Sharma et al., 2018). This new appreciation of the role played by the gut microbiome has resulted in the better understanding of a major intermediary between the brain and the food we consume. The role of the microbiota in regulation of the central nervous system (CNS) and manifestation of psychiatric disorders has recently become an area of intense clinical and preclinical research (Collins and Bercik, 2013; Foster and McVey Neufeld, 2013). Alteration in the bacterial composition of the microbiome has been observed after stress (Hassan et al., 2019; Taylor and Holscher, 2020) and gut dysbiosis (i.e., the imbalance of microbial diversity and loss of beneficial bacteria) can influence reward sensitivity (Hofford et al., 2021), likely controlled by shifts in metabolite levels and immunological function within limbic brain regions (Foster et al., 2017; Meckel and Kiraly, 2019).

The involvement of the brain in mediating these systems has a novel avenue of research. Studies have shown that circumventricular neural substrates, such as the hypothalamus, which control homeostasis, are highly involved in the manifestations of metabolic-mood-like symptoms (McLean et al., 2019; Vagena et al., 2019). Limbic regions, such as the hippocampus (HIP), nucleus accumbens (NAc), and ventral tegmental area (VTA), have also been implicated in the dysregulation caused by chronic stress and/or diet (Décarie-Spain et al., 2018; Lizarbe et al., 2018; Nieh et al., 2016; Wook Koo et al., 2016). These insults result in major changes in second messenger system cascades that subsequently influence the physiological properties of these

mesolimbic circuits (Iñiguez et al., 2010a, 2010b; Warren et al., 2014). However, it is unclear in which cell type many of these changes take place. Classically, bulk tissue containing both neurons and glia are used for biochemical profiling of neural substrates. The lack of resolution and potential to observe no differences as a result of opposing biochemical pathways (e.g., if a target is upregulated in one cell-type while being down regulated in a different adjacent cell-type) within the multicellular milieu of a given brain region calls for the need for more cell-type specific approaches.

The role of microglia, which are highly specialized cells in charge of removal of dead cells and debris in the CNS; in the pathophysiology of the metabolic-mood comorbidity has become of great interest. Microglia have been implicated in their response to stress (Réus et al., 2015) and high-fat diets (Valdearcos et al., 2014), causing neuroinflammation and alterations in the pathways that regulate utilization of energy within the cell (Kalsbeek et al., 2016). Current research has focused on the involvement of microglia in the hypothalamus, a circumventricular brain region that regulates food intake and other motivated behaviors (Valdearcos et al., 2014). Given the sparsity of literature addressing the role of glia within limbic regions such as the NAc and VTA, one of the aims of this dissertation is to determine if neurobiological alterations are mediated through microglia using an adolescent model. Though prevention through behavioral modifications (i.e., healthy diets, exercise) is the greatest weapon in the fight against this comorbidity (Park et al., 2017; Widmer et al., 2015), novel treatments are needed to target neural substrates that may have already been impaired.

The interaction between chronic stress and diet during adolescence is a gap in the literature that may help address the growing issue of metabolic and mood dysfunction. I aim to characterize

some the behavioral and physiological phenotypes that are induced by early-life stress and WSD. I hypothesize that the combination of stress and poor diet in adolescence will result in the rapid progression of long-lasting symptoms that will be mediated by secondary messenger systems in the reward-mediating regions of the brain, the immune system, as well as the gut microbiome. Further analysis of the effects of poor diet compared against concomitant stress may aid in understanding the various processes involved in metabolic-mood syndrome. Ultimately, I hope this research will spur new avenues of investigation, specifically in terms of potential therapeutics to better understand how to maintain long-term functioning for individuals suffering from these comorbid conditions.

CHAPTER II

TEMPORAL CHARACTERIZATION OF BEHAVIORAL, PHYSIOLOGICAL, AND BIOCHEMICAL OUTCOMES AFTER ADOLESCENT EXPOSURE TO HIGH-FAT DIET AND CHRONIC SOCIAL DEFEAT STRESS

Introduction

There exists a strong association between mood dysregulation and diet-related disorders (Marazziti et al., 2014; Pan et al., 2012), with stress playing a pivotal modulating role in the deleterious manifestations of these conditions (Alastalo et al., 2013a; Loria et al., 2014; Weder et al., 2014). Adolescence is a period of enhanced vulnerability to stress, which can precipitate the emergence of major depressive disorder (MDD) and anxiety-related disorders (Eiland and Romeo, 2013), often causing life-long detriments (Kessler et al., 2007; Merikangas et al., 2010). MDD is highly comorbid with obesity and, more broadly, metabolic syndrome (MetS) (van Reedt Dortland et al., 2010; Vogelzangs et al., 2014). Afflicted youth often adopt poor eating habits (Muñoz et al., 1997; O'Neil et al., 2014; Peeters, 2018), deriving sources of energy from foods high in fats and sugars (Reedy and Krebs-Smith, 2010). Such dietary habits are highly concerning given the bidirectional nature of the relationship between MDD and MetS, with each predicting the onset of the other (van Reedt Dortland et al., 2010; Vogelzangs et al., 2014). The symptomatology of mood- and diet-related disorders often overlap and, though well-recognized, the mechanism(s) underlying this comorbidity are not well-understood (Mansur et al., 2015).

Preclinical studies using calorically-dense western-style diets (WSDs), including those high in both fats and carbohydrates, referred to here as high-fat diet (HFD), demonstrate that exposure

to specific WSDs lead to dysregulated body weight and MetS (Zemdegs et al., 2016), as well as anxiety- (Naneix et al., 2017) and depressive-like behaviors (Hassan et al., 2019). Most studies using WSDs to induce obesogenic phenotypes in adult rodents contain 60% kcal from fat, which is significantly higher than the typical human WSD (Speakman, 2019). Dietary Reference Intakes recommend that adolescents consume 25-35% of kcal from fat, 45-65% from carbohydrates, and 10-30% from proteins (Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, 2011). A WSD would exceed >~35% from fats and/or >~65% from carbohydrates. Additionally, the role of fats in the manifestation of these disorders has been an area of intense research for several decades, whereas the contribution of carbohydrates has largely been minimized (Kearns et al., 2018a). Rodent studies utilize very-high fat WSDs to expedite physiological and behavioural deficits; however, it may take 8-20 weeks of consumption for deficits to emerge, resulting in rodents tested in late-adulthood and, at times, geriatric age time-points (Moreno-Fernández et al., 2018).

Despite stress likely being a central factor modulating the comorbidity between MDD and poor diet, its specific role in this context is vastly understudied. In the experimental setting, various stress paradigms have exhibited dysregulated metabolic parameters and altered physiological responses, though the results can vary greatly. Chronic stressors can result in increased (Coccurello et al., 2018; Pecoraro et al., 2004) or decreased (Rabasa et al., 2011; Wang et al., 2012) food intake, increased (Farias-Silva et al., 2002) or reduced (Foster et al., 2006) insulin concentrations, as well as variable changes in blood glucose levels (Farias-Silva et al., 2002); and much of this variation may be due to the nature and magnitude of the specific stressor. The chronic social defeat stress (CSDS) paradigm is an ethologically relevant and robust stressor that produces

metabolic abnormalities that may play a role in the pathogenesis of obesity, such as increases in proinflammatory cytokines (Hodes et al., 2014) and microbiota alterations (Aoki-Yoshida et al., 2016; Bharwani et al., 2016). In some studies, when HFD (45% kcal fats, 35% kcal carbohydrates) is administered after CSDS, mice exhibit dysregulated body weight and lipid synthesis (Chuang et al., 2010a); to contrast, other studies have shown HFD buffering against stress (Finger et al., 2011; MacKay et al., 2017). Furthermore, most of these findings have been derived from adult rodents, forgoing adolescence, a key developmental period in which most of these symptoms often emerge in humans (Kessler et al., 2007; Mojtabai et al., 2016).

Reward dysregulation is one of many shared symptoms between mood and diet-related disorders (Lutter and Nestler, 2009; Volkow et al., 2013). The consumption of WSDs and junk foods has been shown to disturb dopaminergic signalling withing the mesolimbic circuit, which is associated with alteration in the hedonic value of palatable foods (Berridge, 2009; Johnson and Kenny, 2010). These deficits are thought to be long-lived, changing reward sensitivity to appetitive stimuli later in adulthood (Naneix et al., 2018; Vendruscolo et al., 2010). Brain regions known to regulate mood and reward, such as the nucleus accumbens (NAc), ventral tegmental area (VTA), and hippocampus have been altered by both diet (Lutter and Nestler, 2009; Sharma and Fulton, 2013; Teegarden et al., 2009) and stress (Ironside et al., 2018; Krishnan et al., 2008; Yoshida et al., 2021). Secondary messenger systems such as the protein kinase B (*Akt*) and mitogen-activated protein kinase (*Erk1/2*) pathways are known to regulate responses to both drug- and stress-induced changes in reward sensitivity (Alcantara et al., 2014; Iñiguez et al., 2010a, 2010b; Krishnan et al., 2008). Both *Akt* and *Erk* have also been implicated as potential targets for treating obesity, as they play a role in adipose metabolism and inflammation (Balland et al., 2014; Ozaki et al., 2016). The

role of these intracellular signalling markers has not yet been evaluated within the NAc or VTA within the context of early-life stress and WSD exposure. Here, I hypothesize that the synergistic effect of these major insults will result in compounding neurobiological deficits. More specifically, I expect that exposure to stress and HFD will induce changes in secondary messenger signalling that resemble a depressive-like state (Iñiguez et al., 2010a; Krishnan et al., 2008) and that the combination of stress and diet would potential the deleterious effects.

Given the lack of research examining neurobiological outcomes of exposure to stress as well as poor diet during adolescence, I sought to characterize how exposure to either entity would potentially induce behavioral and physiological dysregulation. To this end, I first tested whether timing of adolescent exposure to stress or diet was of importance. To this end, adolescent mice were exposed to CSDS either before or after HFD exposure. Physiological measures such as body weight and caloric intake were measured, and behavioral assays were conducted to assess changes in social and reward-related behaviors. Finally, biochemical analysis of *Akt* and *Erk* within the NAc and VTA was performed to characterize whether stress and HFD exposure leads to neurobiological modifications.

Methods

Animals

Male C57BL/6J mice (Jackson Labs, Bar Harbor, Maine), postnatal day (PD) 28 at time of arrival and CD-1 retired breeders (Charles Rivers, North Carolina) were housed at 23°C in clear polypropylene boxes on a twelve-hour light/dark cycle (lights on at 7AM, lights off at 7PM) and habituated for 7 days. The C57BL/6J mice were group-housed during habituation and moved to single housing at PD35 (the start of experimental manipulations), while the CD-1 mice were singly housed from arrival. This age was selected because it roughly coincides with early adolescence in humans (Abreu-Villaça et al., 2010; Andersen, 2003), a developmental stage of increased vulnerability to stress in which the adoption of poor eating habits and the onset of mood disorders often emerge (Banfield et al., 2016; O'Neil et al., 2014; Paus et al., 2008). Organ collection was performed at the end of the experiments: mice were sacrificed, the hearts and kidneys were rapidly harvested, and wet organ weight was recorded. Experimental procedures were conducted in strict compliance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003) and approved by Texas A&M University's Animal Care and Use Committee.

Stress

Chronic social defeat stress (CSDS) was performed as described previously (Berton and Nestler, 2006; Golden et al., 2011; Iñiguez et al., 2014a) with minor modifications. Adolescent mice were randomly assigned to a daily session of CSDS (10 days) lasting 10 minutes each. Briefly, the home cage of the CD-1 mouse was separated into two compartments by a perforated

clear Plexiglas divider. One side of the compartment temporarily housed the intruder mouse while the other housed the CD-1 retired breeder aggressor. During the daily sessions, the mouse intruder was placed into the territorialized home-compartment of a CD-1 mouse in the cage to the right of the original housing, and subsequently defeated. At the end of the defeat session, the mouse was placed overnight in the compartment adjacent to the CD-1 mouse that socially defeated it. To minimize physical injury, the daily sessions were terminated as soon as the intruder mouse adopted a submissive posture.

Diet

Mice were randomized to receive either standard normal chow (NC; Teklad Rodent Diet; 8604; fat content 14% kcal, carbohydrate content 54% kcal from starches) or high-fat/high carbohydrate diet (HFD; Research Diets; D12451; fat content 45% kcal from lard, carbohydrate content 35% kcal from sucrose; Table 2) ad libitum. The diet was placed in metal food hoppers and weighed daily. The hoppers were removed prior to CSDS and placed into a new cage with its respective mouse after CSDS. Caloric intake was determined from the weight of food consumption and converted to adjusted caloric intake by normalizing to the body weight of the respective mouse (kcal consumed/g of body weight).

Social Interaction Test

The social interaction test (SIT), a behavioral assay assessing social avoidance, is used as a readout to validate the CSDS paradigm. Each SIT was performed 24 hours after the last defeat session. Briefly, the SIT is composed of two-sessions. In the first session, a mouse is allowed to explore an open field arena (40 cm × 40 cm) for 2.5 minutes. Along one side of the arena is a wire mesh cage that remains empty during the first trial (i.e., no social target present). This mouse is

then removed, and a novel CD-1 mouse is placed into the wire mesh cage (i.e., social target present). The test mouse is then brought back into the arena and the amount of time it spends in the "interaction zone" (8 cm wide corridor surrounding the cage), as well as the time spent in the "corners" farthest from the mesh cage, are measured during the second 2.5-minute trial (social target present). Socially defeated mice explore the interaction zone significantly less when another mouse is present, spending more time in the corners. Interaction ratios below 1.0 indicate social avoidance and susceptibility to stress (Berton and Nestler, 2006; Warren et al., 2013).

Conditioned Place Preference

Place preference conditioning was carried out as previously described (Alcantara et al., 2014), using a three-compartment apparatus, where compartments differed in floor texture and wall coloring. On the preconditioning day, mice were allowed to freely explore the entire apparatus for 30 minutes to obtain baseline preference to any of the three compartments (side compartments: 23 × 16 × 36 cm; middle compartment: 9 × 16 × 36 cm, L × W × H). Conditioning trials (30 minutes, two per day) were given on three consecutive days. During the conditioning trials, mice received a subcutaneous saline injection (2 ml/kg) and were confined to a compartment of the apparatus (unbiased procedure). After 4 hours, mice received morphine (0.5 or 1 mg/kg, s.c.) and were confined to the opposite side compartment. On test day (preference), mice were again allowed to freely explore the entire apparatus for 30 minutes. Data are shown as time spent in drug paired side – time spent in the non-drug paired side.

Sucrose and Saccharin Preference Test

This test consisted of a two-bottle choice paradigm in which mice were given a choice between consuming water versus either sucrose (Research Products International: S24060-5000),

or saccharin (Sigma-Aldrich: 240931). This paradigm has been used extensively to assess the effects of stress-induced anhedonia (Papp et al., 1991; Warren et al., 2011). Mice were habituated to drink water from two bottles over two nights. After 24 hours of ad libitum access, the bottle positions were switched (left to right and vice versa) to avoid side preferences and left overnight. Mice were exposed to water and either sucrose (0.125%, 0.25%, 0.50%, 1%, 2.5%, 5%, 10%) or saccharin (0.0005, 0.005, 0.05, 0.5%) for 48 hours per concentration, and the bottle was measured, and its position switched. The preference for sucrose, or saccharin, over water [e.g., sucrose/ (sucrose + water)] was used as a measure of sensitivity to reward. This formula considers the intake of fluid, so any effects are independent of differences in total fluid intake.

RT-qPCR

Mice were sacrificed 24 hours after the last day of sucrose preference. Bilateral punches were taken from the NAc (14 gauge) and VTA (15 gauge) and stored at -80°C until use. RNA was isolated using RNEasy Micro kits (QIAGEN) and cDNA was created from these samples using iScript cDNA synthesis kits (Bio-Rad). Quantitative real-time reverse transcription-PCRs (qPCRs) were performed in triplicate using 96-well PCR plates and SYBR Green MasterMix (Thermo Fisher) with a Bio-Rad CTX Connect 384 according to manufacturer's instructions. Threshold cycle [C(t)] values were measured using the supplied software and analyzed with the ΔΔC(t) method, as described previously (LaPlant et al., 2010; Vialou et al., 2010). Primer sequences for *Erk1*, *Erk2*, *Akt*, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are listed in Table 1.

Statistical analyses

Data were analyzed using GraphPad Prism (version 9) software. Changes in body weight, food consumption, and adjusted caloric intake were compared using analysis of variance

(ANOVA), followed by Šidák post-hoc tests with stress (PS), diet (NC, HFD), and time (averaged 7 days across week: repeated measure) as sources of variance. When appropriate, two-way ANOVAs or Student's t-tests were used to determine statistical significance of pre-planned comparisons. Data are expressed as the mean ± SEM, with statistical significance set at $p<0.05$.

Results

5 weeks of adolescent HFD exposure on body weight, food consumption, adjusted caloric intake, and social interaction.

Experimental Design. After habituation, adolescent male C57 mice were moved from grouped to individual housing and randomly assigned to either the NC (n=8) or HFD (n=8) conditions to assess the effects of HFD on body weight, food consumption, adjusted caloric intake, and social interaction (SI). The mice were exposed to 5 weeks (PD35-69) of diet before measuring SI as depicted in Figure 1*A*.

Body Weight. Body weight was measured daily and averaged across the week to yield weekly group averages (Fig. 1*B*). A two-way repeated measures ANOVA showed that mice in both the NC and HFD conditions gained weight over time ($F_{(2,39)}$= 147.4; p<0.0001) but did not differ from each other as a function of diet exposure ($F_{(1,14)}$ = 0.09; p>0.05). No differences in weight gain between groups were detected throughout the 5 weeks (p>0.05).

Food Consumption. Total food consumption was measured daily and averaged as described (Fig. 1*C*). Significant changes in food consumption were a factor of both time ($F_{(2,30)}$= 20.92; p<0.0001) and diet exposure ($F_{(1,14)}$= 512.5; p<0.0001). Between-group analysis demonstrated that HFD-exposed mice consumed significantly less food compared to the NC-exposed group across the 5 weeks (p<0.05), indicating that the HFD-exposed mice feeding behavior was homeostatically regulated, leading to less consumption of the more calorically dense diet. Interestingly, within-group comparisons indicated that mice in both groups consumed significantly more chow only during the first week (p<0.05), an effect that may have been the result of novelty-induced hyperphagia in response to the novel diet.

Adjusted Caloric Intake. Weekly caloric intake was measured as described, then divided by their respective body weight and averaged across each week to depict the adjusted caloric intake (Fig. 1*D*). Differences in adjusted caloric intake were a function of time ($F_{(2,37)}$= 11.79; p<0.0001) but not diet ($F_{(1,14)}$= 0.97; p>0.05). This difference is underlined by an adjusted caloric consumption in both groups in week 1 that is significantly higher as compared to the remaining four weeks (p<0.05). Overall, these findings show that 5 weeks of HFD does not yield short-term (1-month) effects on caloric intake or body weight, except for the first week of diet exposure.

Social Interaction. The effect of NC and HFD exposure on social interaction was examined and represented by an interaction ratio. An unpaired *t*-test revealed no significant differences between the groups ($t_{(14)}$=1.672; p>0.05), indicating that 5 weeks of HFD alone had no effect on social interaction (Fig. 1*E*).

5 weeks of HFD exposure during adolescence has no effect on morphine preference.

Adolescent mice were habituated for one week and subsequently exposed to 4 weeks of NC or HFD and tested for conditioned place preference for subthreshold doses of morphine (SAL; 4/group, MOR 0.5 or 1mg/kg; 5/group; Fig. 2*A*). Time spent in the drug-paired compartment was subtracted from time spent in non-drug paired compartment (Fig. 2*B*). Two-way ANOVA shows a significant interaction between dose and diet ($F_{(2, 22)}$= 6.505, p<0.006). The NC-exposed mice showed a significant preference for 1 mg/kg of morphine compared to saline. Post-hoc analysis revealed that HFD-exposed mice do not show a preference for either dose of morphine compared to their respective saline controls; however, the highest morphine dose (1 mg/kg) was significantly

different compared to the NC+SAL-exposed mice. Neither group showed a preference for the lowest morphine dose (0.5 mg/kg).

Exposure to 2 weeks of HFD before CSDS leads to increases in weight gain.

Experimental design. After the habituation period, the mice were moved into single housing (PD35) with NC or HFD (n=12/group) available ad libitum for 8 weeks (PD35-77). By the end of the first 2 weeks of diet exposure, the mice were exposed to CSDS (PS) for 10 days, followed by the SIT 24 hours after the last defeat exposure (Fig. 3*A*).

Body weight. After CSDS exposure, daily body weight measurements continued for ~6 weeks and were averaged as described (Fig. 3*B*). A mixed-model repeated-measures ANOVA revealed that body weight changed as a function of diet exposure (between-group main effect: $F_{(1,22)}$=12.63; p<0.0018) and time (within-subject main effect: $F_{(1,37)}$=248.8; p<0.0001). Post-hoc analyses indicated that the HFD+PS-exposed mice displayed higher body weights by week 4, a week after CSDS exposure, when compared to the NC+PS-exposed group (p<0.0056). By the last week of the diet (week 8), the HFD+PS-exposed mice remained heavier than the NC+PS-exposed mice (p<0.0027). These findings demonstrate that a stressful period during HFD exposure can induce increases in body weight as early as one week after experiencing the stress, an effect that is maintained 6 weeks after the stressor.

Food consumption. Food intake was measured as previously described across 8 weeks (Fig. 3*C*). A mixed-model repeated-measures ANOVA revealed that food consumption was influenced by diet (between-group main effect: $F_{(1,22)}$=178.3; p<0.0001) and time (within-subject main effect: $F_{(2,52)}$=7.52; p<0.0008). The HFD+PS-exposed mice consumed fewer grams of food as compared

to the NC+PS-exposed mice beginning from week 2 and continuing through week 8 ($p<0.0001$), demonstrating, as previously stated, that mice in the HFD condition consumed less of the calorically dense diet as a form of self-regulation.

Adjusted caloric intake. Caloric intake across the 8 weeks was measured and converted to adjusted caloric intake (i.e., kcal consumed per gram of body weight) as described (Fig. 3*D*). A mixed-model repeated-measures ANOVA demonstrated that adjusted caloric intake was influenced by diet ($F_{(1,22)}=14.35$; $p<0.001$) and time ($F_{(2,47)}=138.1$; $p<0.0001$). Post-hoc analyses indicated that HFD+PS-exposed mice consumed more calories during the first week ($p<0.0001$) as compared to the NC+PS-exposed mice. No significant differences between the groups during any of the other weeks were detected.

Social interaction. The impact of NC or HFD before CSDS on social interaction was examined. A parallel second group of mice (n=5), no-diet/no-stress, was used as controls (CON) for the SIT (Fig. 3*E*). One-way ANOVA showed significant differences in social interaction as a function of stress ($F_{(2,26)}=8.819$; $p<0.0012$), indicating that the NC- and HFD-exposed mice avoided the social target (a depression-like phenotype) as differences in interaction ratio between the diet groups were detected ($p>0.05$).

Exposure to 2 weeks of WSD before CSDS induces deficits in sucrose preference in adulthood.

Changes in reward sensitivity were assessed 2 weeks after exposure to CSDS in the same cohort of mice reported in Figures 3-4, using the sucrose preference test during adulthood PD60-77 (Fig. 4*A*). A mixed-model repeated-measures ANOVA showed that differences observed in sucrose preferences were a factor of both diet ($F_{(1,22)}=56.70$; $p<0.0001$) and sucrose concentration

($F_{(3,74)}$=66.82; p<0.0001; Fig. 4*B*). Post-hoc analyses revealed that mice in the HFD condition drank less sucrose than the NC-exposed mice after PS exposure at 0.25, 0.5, 1, 2.5, 5, and 10% sucrose concentrations (p<0.05, respectively).

Exposure to 2 weeks of HFD before stress leads to dysregulation of secondary messenger systems within the mesolimbic system.

Quantitative rt-PCR was used to assess gene expression changes between NC+CSDS- (n=8) and HFD+CSDS-exposed (n=7) mice (Fig. 5*A*) Within the NAc, *Akt* expression was decreased in the HFD+CSDS-exposed mice (Fig. 5*B*; $t_{(13)}$=3.268; p<0.01), whereas *Erk1* ($t_{(13)}$=2.24; p<0.05; Fig. 5*C*) was upregulated. There was no significant change in *Erk2* ($t_{(13)}$=1.05; p>0.05; Fig. 5*D*). Within the VTA, there was no differences in *Akt* expression ($t_{(13)}$=0.61; p>0.05; Fig. 5*E*), and both *Erk1* ($t_{(13)}$=3.255; p<0.05; Fig. 5*F*) and *Erk2* ($t_{(13)}$=2.747; p<0.05; Fig. 5*G*) were significantly downregulated.

Effects of CSDS exposure followed by HFD on social interaction, weight gain, and caloric intake.

Experimental design. Adolescent mice were randomly assigned to the control (CON; n=6) or CSDS conditions (n=24/group), followed by a SIT 24 hours later. After the SIT, only the CSDS-exposed mice were further randomly separated into NC- or HFD-exposed groups (n=12/group; Fig. 6*A*).

Social interaction. An unpaired *t*-test revealed that CON- and PS-exposed mice had significantly different interaction ratios ($t_{(28)}$=7.350; p<0.0001), demonstrating higher social avoidance by the PS-exposed mice (Fig. 6*B*).

Body weight. Body weights were measured for 5 weeks following CSDS. Body weights were measured daily and averaged as described (Fig. 6*C*). A mixed-model repeated-measures ANOVA indicated that changes in body weight were a function of diet exposure (between-group main effect: $F_{(1,22)}$=19.36; p<0.0002) and time (within-subject main effect: $F_{(1,39)}$=94.75; p<0.0001). Post-hoc analyses indicated that the PS+HFD-exposed mice displayed higher body weights by week 2 when compared to the PS+NC diet controls (p<0.05). By the last week of diet exposure (week 5), the PS+HFD-exposed mice remained heavier than the PS+NC-exposed group (p<0.05). Interestingly, the PS+HFD-exposed mice showed weight gain sooner (week 2) than the PS+NC-exposed mice, which demonstrated weight gain by week 4 (Fig. 6*C*).

Food intake. Food intake was measured as previously described across the 5 weeks (Fig. 6*D*). A mixed-model repeated-measures ANOVA revealed that food consumption varied as a function of diet (between-group main effect: $F_{(1,22)}$=246.3; p<0.0001) and time (within-subject main effect: $F_{(2,61)}$= 30.96; p<0.0001). Post-hoc analyses showed that the PS+HFD-exposed mice consumed fewer grams of food as compared to the PS+NC-exposed mice across the 5 weeks (p<0.05).

Adjusted caloric intake. Caloric intake across the 5 weeks was measured and converted to adjusted caloric intake as described (Fig. 6*E*). A mixed-model repeated-measures ANOVA revealed that adjusted caloric intake was influenced by time (within-subject main effect: $F_{(2,58)}$=142.1; p<0.0001). Post-hoc analyses revealed that the PS+HFD-exposed mice consumed

more calories during the first week only as compared to the PS+NC-exposed group ($p<0.05$). No significant differences between the groups during any of the other 4 weeks were detected.

Effects of CSDS exposure followed by HFD on preference for saccharin.

A separate group of adolescent mice (n=8/group) were used to further assess whether exposure to PS+HFD would induce dysregulation in reward sensitivity as observed after CSDS (Fig. 7*A*). To this end, a two-bottle saccharin preference test was performed with varying concentrations of saccharin (Fig. 7*B*). A repeated-measures ANOVA exhibited differences in saccharin preferences as a factor of diet ($F_{(1,14)}=26.90$; $p<0.01$), saccharin concentration ($F_{(2,29)}=12.47$; $p<0.01$), as well as an interaction between the two ($F_{(3,42)}=5.790$; $p<0.01$). Post-hoc analyses revealed that PS+HFD-exposed mice showed decreases in saccharin preference at 0.05% and 0.5% concentrations when compared to their respective PS+NC-exposed group ($p<0.05$). These findings indicate that sensitivity to reward is dysregulated by concurrent stress and WSD independent of the caloric value of the sweetened solution.

Effects of CSDS exposure followed by HFD on heart and kidney weight.

The same mice from Figures 7-8 were sacrificed and their hearts and kidneys were harvested and weighed. There were no differences in heart weight ($t_{(22)}=0.139$; $p>0.05$; Fig. 8*A*), but there was a significant increase in kidney weight in the PS+HFD- compared to PS+NC-exposed mice ($t_{(22)}=1.757$; $p<0.05$; Fig. 8*B*).

Discussion

A bidirectional relationship exists between neuropsychiatric disorders, such as major depression and post-traumatic stress disorder, and diet-related disorders like metabolic syndrome (MetS) and cardiovascular disease (CVD) (Harris and Barraclough, 1998; Luppino et al., 2010; Marazziti et al., 2014; Pan et al., 2012; Thombs et al., 2006), with stress as a major modulating factor in the development of obesity in both human and animal models (Goto et al., 2014; Karatsoreos et al., 2010; Tajik et al., 2014; De Vriendt et al., 2009). Despite the prevalence and negative consequences of these comorbid conditions, the paucity of evidence delineating the specific neurobiological interactions between stress, metabolic dysregulation, and mood disorders is surprising. The lack of data surrounding this comorbidity is especially prevalent regarding adolescence, a developmental stage distinguished by an increased vulnerability to stress and the initial development of mood disorders and poor eating habits (Banfield et al., 2016; O'Neil et al., 2014; Paus et al., 2008). To fill in the gaps in our knowledge, I sought to demonstrate the potential detrimental effects of early-life stress and western-style, high-fat and high-carbohydrate diets (HFD) on physiology, reward sensitivity, and neurobiological alterations.

In this study, I first sought to determine whether five weeks of ad libitum consumption of HFD with only ~45% kcal during the sensitive period of adolescence would be sufficient to induce significant changes in weight gain and caloric intake in male mice. Although adolescent mice in both the normal chow (NC) and the HFD conditions gained weight over time, as expected, this did not differ between the groups across the four weeks. This lack of weight gain is a common feature observed in adult rodents, as it may take several months to appreciate significant weight gain (Moreno-Fernández et al., 2018; Speakman, 2019; van der Heijden et al., 2015). Despite no

significant changes in weight gain noted between the diet groups, the mice in the HFD condition consumed significantly less of the calorically-dense diet compared to the NC-exposed controls across the 5 weeks, indicating that the consumption of the HFD-exposed mice was homeostatically regulated. This finding was corroborated by the observation of similar adjusted caloric intake between the groups (see Fig. 1*D*). This phenotype does not reflect human adolescent behavior in which environmental and social cues can promote overeating palatable diets (Hetherington and Blundell-Birtill, 2018; Reichelt and Rank, 2017). Together, these findings indicate that 5 weeks of HFD exposure did not yield significant effects on body weight or caloric intake, except during the first week of diet exposure, an effect that may have been driven by novelty-induced hyperphagia in response to the mice being moved into single-housing (Schipper et al., 2018, 2019).

Social avoidance is a symptom of major depressive disorder (Fernández-Theoduloz et al., 2019) and obesity (Hommel et al., 2010; Phillips et al., 2012) and is considered a maladaptive anhedonic behavior for intrinsically social mice. To examine the long-term behavioral effects of diet consumption, I assessed social interaction in NC- and HFD- exposed mice; there was no significant difference between the NC and HFD groups, indicating no changes in social behavior. However, there was a trend towards reduced interaction in HFD-exposed mice that could indicate that consumption of HFD long-term through adolescence leads to social deficits in adulthood. Intriguingly, studies have shown correlation between body mass index (BMI) and social avoidance (Hommel et al., 2010; Phillips et al., 2012), so it is not without reason to believe that if there had been more drastic weight gain, it would be associated with lower interaction ratios.

The lack of physiological changes in naïve adolescent mice in response to HFD, is not unheard of, as clinically, there is a growing population of young adults that are not definitionally

overweight but do present with metabolic abnormalities associated with obesity (Foulis et al., 2020). These individuals are at a higher risk for diet-related disorders such as CVD later in life (Brown et al., 2020). I hypothesized that despite the lack of weight gain in the adolescent mice as a function of diet, there may be other deficits (i.e., reward insensitivity) present that are similar to those observed in clinically obese patients such as reward regulation dysfunction (Blum et al., 2014; García-García et al., 2013). To this end, I chose to test if adolescence mice exposed to 4 weeks of HFD has altered reward response to a common drug of abuse, morphine. I utilized the conditioned place preference (CPP) paradigm to test if two different low doses of morphine (0.5 and 1 mg/kg) would retrain their rewarding properties after diet exposure. NC-exposed mice showed a preference for 1 mg/kg of morphine but not 0.5 mg/kg; this is consistent with findings from the literature, as doses of 1-80 mg/kg of morphine will typically induce CPP (Sala et al., 1992). Curiously, HFD-exposed mice conditioned to 1 mg/kg of morphine did not show significant preference compared to their respective control group (HFD-fed SAL injected mice) but showed significant preference compared to NC-SAL mice. My hypothesis was partially supported as there was an interaction between diet and dose, indicating reward dysfunction. This data supports literature findings that HFD-exposure is likely influencing limbic regions of the brain altering the response to other appetitive stimuli (Naneix et al., 2017).

Given the numerous detrimental effects of poor diet in children and adolescents (Fryar et al., 2018; Nehus and Mitsnefes, 2019; Weiss et al., 2004) and the known metabolic abnormalities induced by early-life stress reported in the literature (Farr et al., 2015; Gonzalez-Bulnes et al., 2016), I sought to assess the potential negative effects of a stressor, namely, chronic social defeat stress (CSDS; also referred to as physical stress, PS), paired with HFD exposure. Some studies

have yielded inconsistent findings when assessing the effects of stress and HFD in that exposure to HFD before, or during stress exposure, has been shown to buffer against the negative effects of stress (Finger et al., 2011; MacKay et al., 2017). Thus, to determine whether the timing of stress and HFD exposure would influence physiological responses to the other, adolescent mice were exposed to either 2 weeks of HFD before CSDS, or to HFD immediately after CSDS. Mice exposed to 2 weeks of HFD before stress showed increased weight gain within 4 weeks of HFD (1 week after CSDS exposure), when compared to the NC+PS-exposed mice. Although social avoidance was expected in both stressed groups, the magnitude of the avoidance in the HFD-exposed groups was expected to be higher as high-fat diets have been reported to enhance susceptibility to PS (Goto et al., 2016). Though social interaction ratios did not differ, it is interesting to note that the corner ratio, an index of social avoidance (Appendix A1), was significantly higher in the HFD+PS-exposed mice when compared to the CON and NC+PS-exposed groups, perhaps indicating a compounded avoidance effect in the HFD+PS-exposed group.

Changes in reward sensitivity have also been reported as a function of stress and HFD exposure. In this study, reward sensitivity was blunted in the mice pre-exposed to HFD as they exhibited an attenuation of sucrose consumption at 0.25%-10% concentrations, results indicative of both reward deficits and anhedonic behavior (Liu et al., 2018). The mechanism(s) underlying these effects are not known; however, previous work has demonstrated that intracellular signaling mechanisms in the limbic regions of the brain regulate mood and reward, specifically the nucleus accumbens (NAc) and ventral tegmental area (VTA) (Alcantara et al., 2014; Iñiguez et al., 2010a; Krishnan and Nestler, 2011). Within these substrates, kinases such as protein kinase B or *Akt*, a known regulator of stress susceptibility (Krishnan et al., 2008) and extracellular signal-regulated

protein kinase *Erk1/2*, a downstream effector of the brain derived neurotrophic factor (BDNF), regulate reward sensitivity and mood-related behaviors (Iñiguez et al., 2010a, 2014a). I found that the HFD-PS-exposed mice had reduced *Akt* and increased *Erk1/2* mRNA expression in the NAc and nearly the opposite findings within the VTA: no changes in *Akt*, but significantly reduced *Erk1/2* expression. I hypothesized that HFD-PS-exposed mice would show a biochemical profile similar to those seen in stressed mice. These effects are unexpected, as typically *Erk1/2* are upregulated within the VTA in stressed mice (Iñiguez et al., 2010a) and *Akt* is downregulated (Krishnan et al., 2008). The neurobiological basis of these results is unknown and it is very likely that these findings may have been confounded by the 2 weeks of sucrose preference testing the mice went through before brain sectioning. Data from our lab has previously demonstrated that viral-mediated upregulation of *Erk2* in the VTA is associated with an increase in sucrose preference despite induction of other depressive-like behaviors (Iñiguez et al., 2010a). It is plausible that *Erk2* is regulated differently by stress and appetitive stimuli such as sucrose or is sensitizing the reward circuit (Yap and Miczek, 2008).

To test whether the administration of stress prior to diet influences mood-related behaviors and metabolic parameters, mice were subjected to CSDS before HFD. I found that the PS+HFD-exposed mice gained weight, but this change was accelerated, becoming apparent as early as week two of HFD exposure, compared to their respective NC+PS-exposed controls. This phenomenon has recently been shown in adult mice exposed to repeated social isolation in which their paws are submerged in cold water for 1 hour, resulting in rapid increases in food intake and subsequent weight gain in the HFD condition (Ip et al, 2019). Interestingly, the rapid weight gain present here is independent of food or caloric intake, as the HFD-exposed mice consumed fewer grams of the

calorically-dense diet, regardless of stress condition, and demonstrated no differences in adjusted caloric intake despite the novelty-induced hyperphagia observed following single-housing. Because stress is typically thought to decrease food intake (E. Morley et al., 1983) and weight gain (Ip et al., 2019; Warren et al., 2013), the lack of change in caloric intake observed in adolescents is intriguing given their rapid increase in body weight. The mechanism(s) underlying this phenomenon is currently unknown. In adult mice, increases in caloric intake of palatable diets is observed when first exposed to social stress (Coccurello et al., 2018; Hassan et al., 2019). Given that CSDS exposure can cause alterations in lipid regulation and energy portioning (Chuang et al., 2010b), it is possible that shifts in metabolic processing are occurring more drastically in adolescence after stress exposure, resulting in rapid weight gain despite consuming the same number of calories as the mice in the NC condition. Involvement of hormones, such as corticosterone (cortisol in humans) produced in the adrenal glands (Marieb and Hoehn, 2007; Taves et al., 2011), may be influencing the manifestation of these physiological deficits given their extensive involvement in various metabolic processes (e.g., glucose utilization and inflammation) (Karatsoreos et al., 2010). Given that adrenocorticotropic hormone regulates the production of corticosterone and is directly correlated with kidney weight in rodents (Akana et al., 1983), I sought to determine if stress and HFD exposure led to change in kidney weight. My data indicates that the PS-HFD-exposed mice exhibit kidney hypertrophy (Fig. 8), a phenomenon also observed in adult mice fed HFD, which is associated with kidney damage (Cheng et al., 2019). Further studies are needed to explore the direct involvement of hormones like corticosterone and determine whether altered cellular respiration pathways during adolescence are influencing the storage and metabolism of macronutrients from different sources in a preferential manner.

The effects of stress followed by HFD exposure on behavioral responsiveness to reward was assessed using saccharin preference. To confirm whether this phenotype persists in mice initially exposed to CSDS before HFD and to ensure the results were not confounded by the calorically dense HFD, a two-bottle saccharin preference test, an artificial sweetener devoid of calories, was performed. Similar to the HFD+PS-exposed mice in the sucrose preference test, a significant reduction in saccharin preference was observed, indicating that the effects of PS+HFD remained, regardless of the timing of stress or diet introduction. The mechanistic underpinning(s) of this effect is not understood. Given the pivotal role played by the mesolimbic dopamine (DA) system with regards to food consumption and reward (Volkow et al., 2017; Wise, 2013), it is conceivable that HFD and stress exposure compromise the dopaminergic reward system. Regions such as the nucleus accumbens (NAc) and the ventral tegmental area (VTA) are involved in regulating motivated behavior and responses to drugs of abuse, stress and natural rewards, making these likely culprits in the dysregulated pathways underlying the mood- and metabolically-influenced behaviors (Bolaños and Nestler, 2004; Di Chiara and North, 1992; Kelley and Berridge, 2002; Wallace et al., 2008).

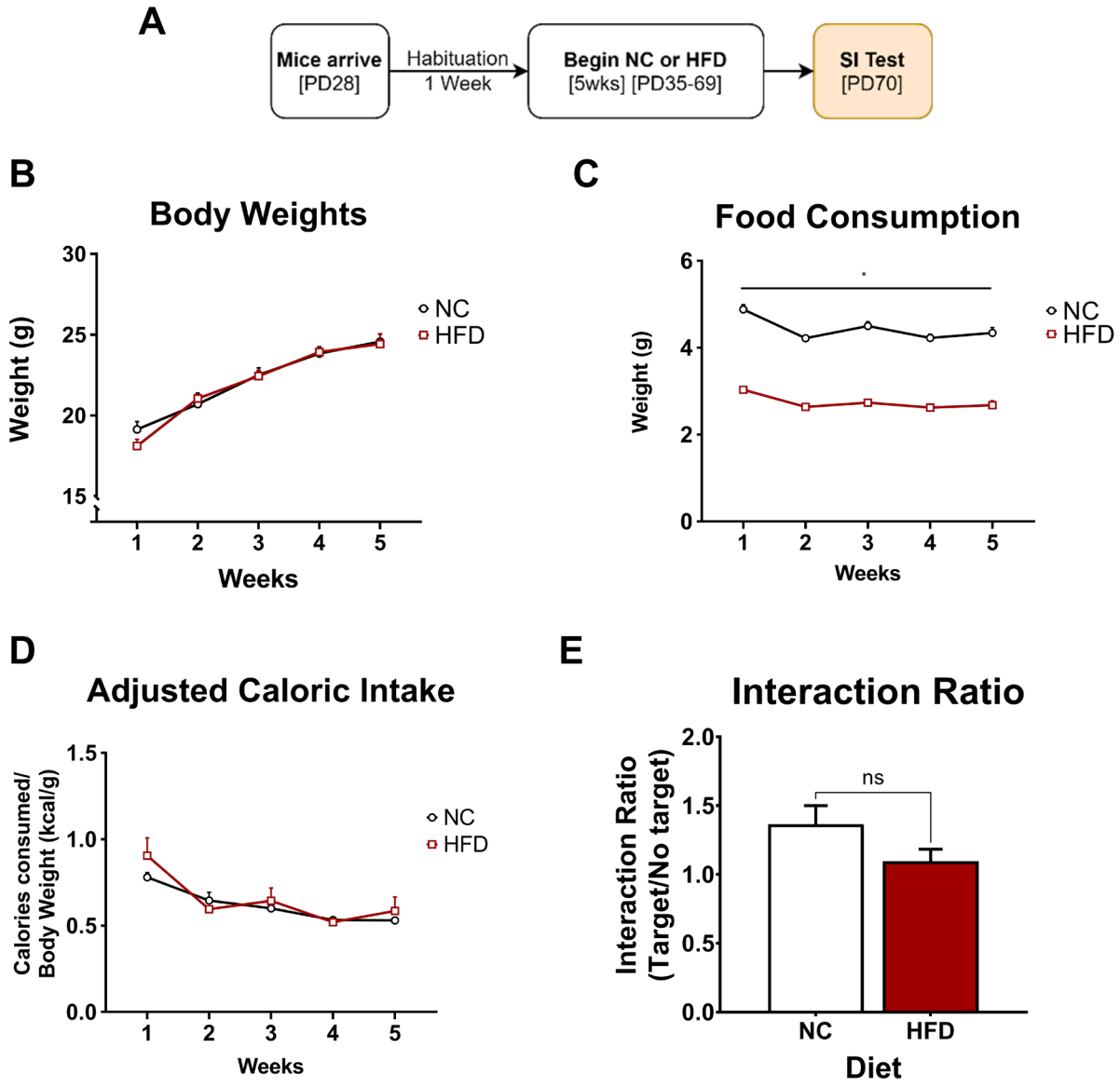
One limitation of this study lies in the use of male subjects only. Studies have indicated that women are more likely to suffer from depression and are at a higher risk of developing obesity after a stressful event (Mannan et al, 2016). The stress paradigm utilized in this study made it not possible to use females as successfully encouraging males to aggressively charge female mice is an ongoing obstacle in the attempt to create a paradigm in which females are exposed to physical stress. Although some approaches have included optogenetic manipulations in transgenic mice, utilization of more aggressive strains of female mice, and the use of male pheromones to incite

males to show aggression towards females (Harris et al, 2018; Newman et al, 2019; Takahashi et al, 2017), these new models have not yet been fully characterized, which will require comprehensive testing to assess their true efficacy in adolescent rodents. Another limitation is the lack of a micronutrient- and protein-controlled chow. The normal chow used in our study is what is commonly given to rodents in many laboratories across the country, though using a matched control chow would have excluded the possibility of off-target effects.

Ultimately, these findings demonstrate that WSD exposure during adolescence leads to physiological and reward-related deficits (i.e., anhedonia) that may lead to the development of maladaptive behaviors and negative health outcomes in adulthood. Here, I have described a paradigm that can elicit a rapid obesogenic-like phenotype, along with deficits in reward and antidepressant responses. Elucidating the relationship between MDD and MetS may uncover crucial implications for adolescent health and sociocultural patterns of behavior for future adult functioning. These types of studies will be crucial in developing a better understanding of the neurobiological interactions between mood and metabolic disorders, as environmental insults like social stress and unhealthy diets can cause long-lasting behavioral and physiological deficits that persist into adulthood (Lobstein et al, 2004).

Figures

Figure 1: 5 weeks of adolescent HFD exposure on body weight, food consumption, adjusted caloric intake, and social interaction.

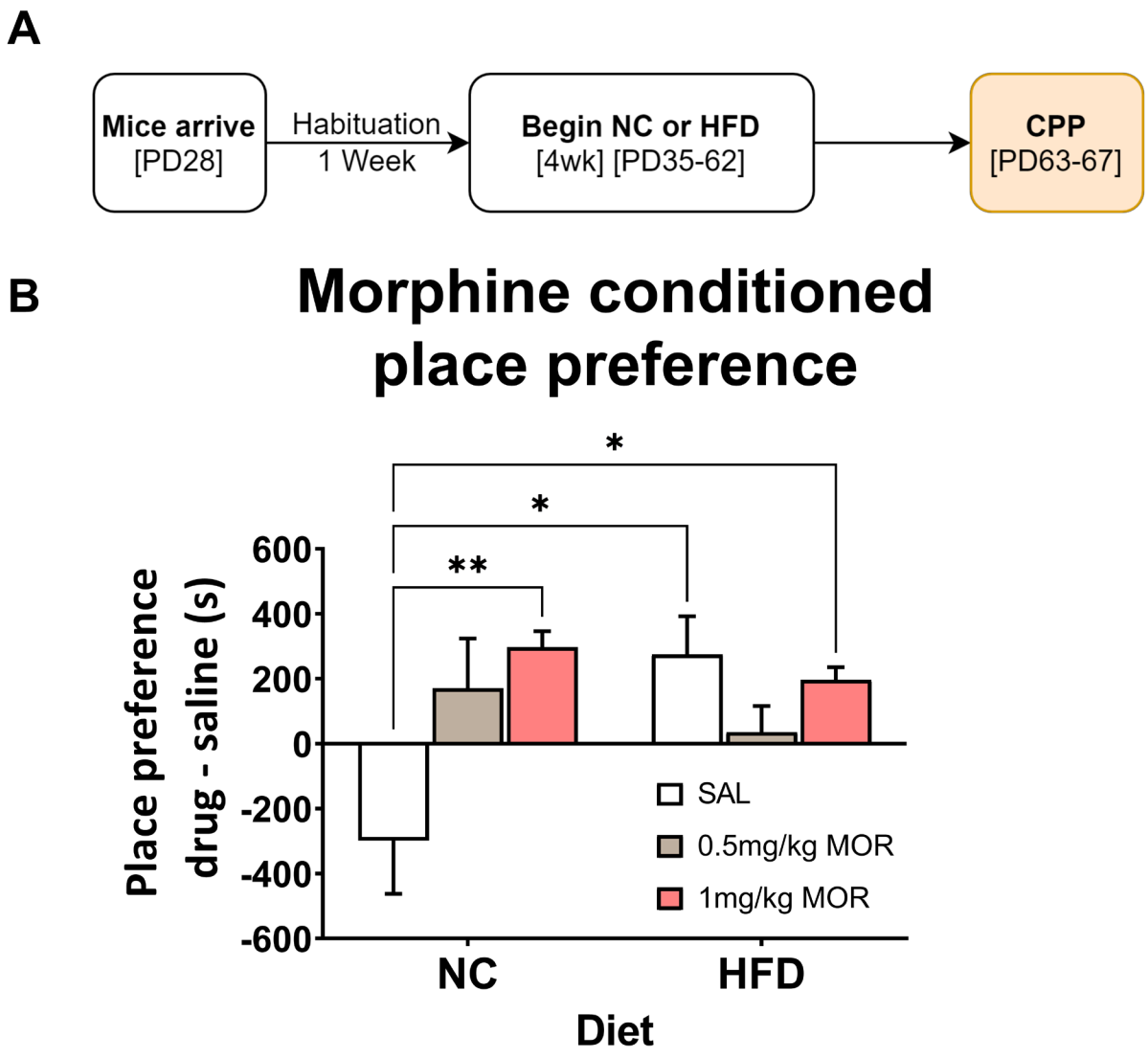


A) Adolescent mice were habituated for one week and subsequently exposed to 5 weeks (PD35-69) of NC (n=8) or HFD (n=8) and tested for social interaction on PD70. ***B)*** The mice in both the NC and HFD conditions gained weight over time ($p<0.001$) without an effect of diet ($p>0.05$). ***C)***

The HFD-exposed mice consumed less of the calorically dense diet compared to the NC-exposed mice across the 5 weeks ($^{*}p<0.05$), indicating that the feeding behaviors of HFD-exposed mice were homeostatically regulated. ***D)*** Each group showed a significant increase in the adjusted caloric consumption in week 1 compared to the rest of the weeks ($^{*}p<0.05$: week 1 vs week 2), without significant differences between the two groups ($p>0.05$). ***E)*** There was no significant difference in social interaction between the NC- and the HFD-exposed mice ($p>0.05$).

$^{*}p<0.05$: significantly different from NC. All data are expressed as the mean ± SEM.

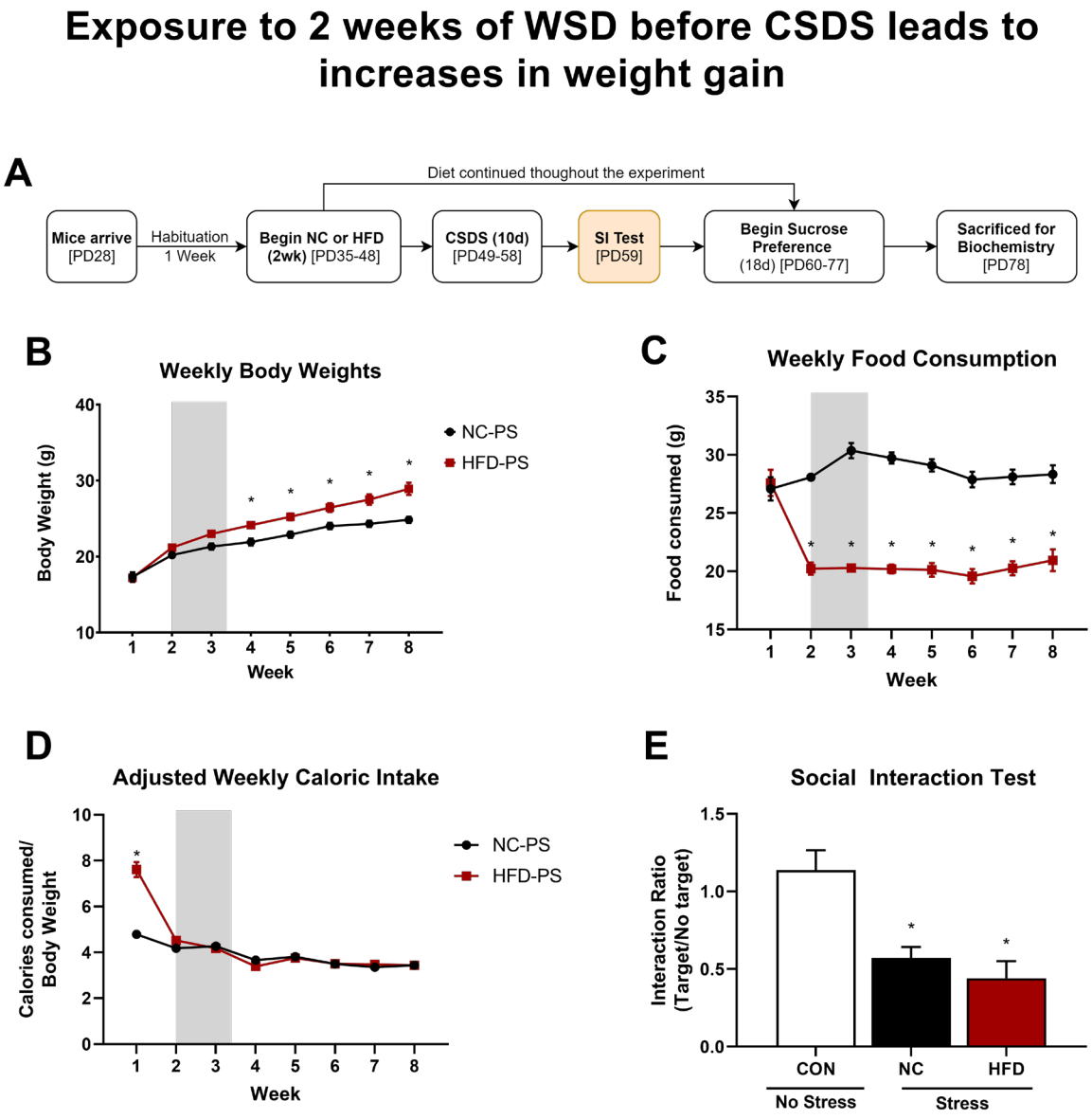
Figure 2: 5 weeks of HFD exposure during adolescence has no effect on morphine preference.



A) Adolescent mice were habituated for one week and subsequently exposed to 4 weeks of NC or HFD and then tested for conditioned place preference to morphine (SAL; 4/group, MOR 0.5mg/kg; 5/group, MOR 1mg/kg; 5/group). **B)** Adolescent (PD30) male C57BL mice were given ad libitum access to HFD for 30 days (PD30-62). A two-way ANOVA demonstrates a significant interaction between dose and diet ($p<0.006$). NC-exposed mice exhibited significant preference for 1 mg/kg

of morphine compared to saline. Post-hoc analyses revealed that HFD-mice do not show a preference for either dose of morphine compared to their respective saline controls; however, the highest dose (1 mg/kg) was statistically significant when compared to the NC-SAL-treated mice. Neither group showed a preference for the lowest dose of morphine (0.5 mg/kg). $^{*}p<0.05$; $^{**}p<0.01$

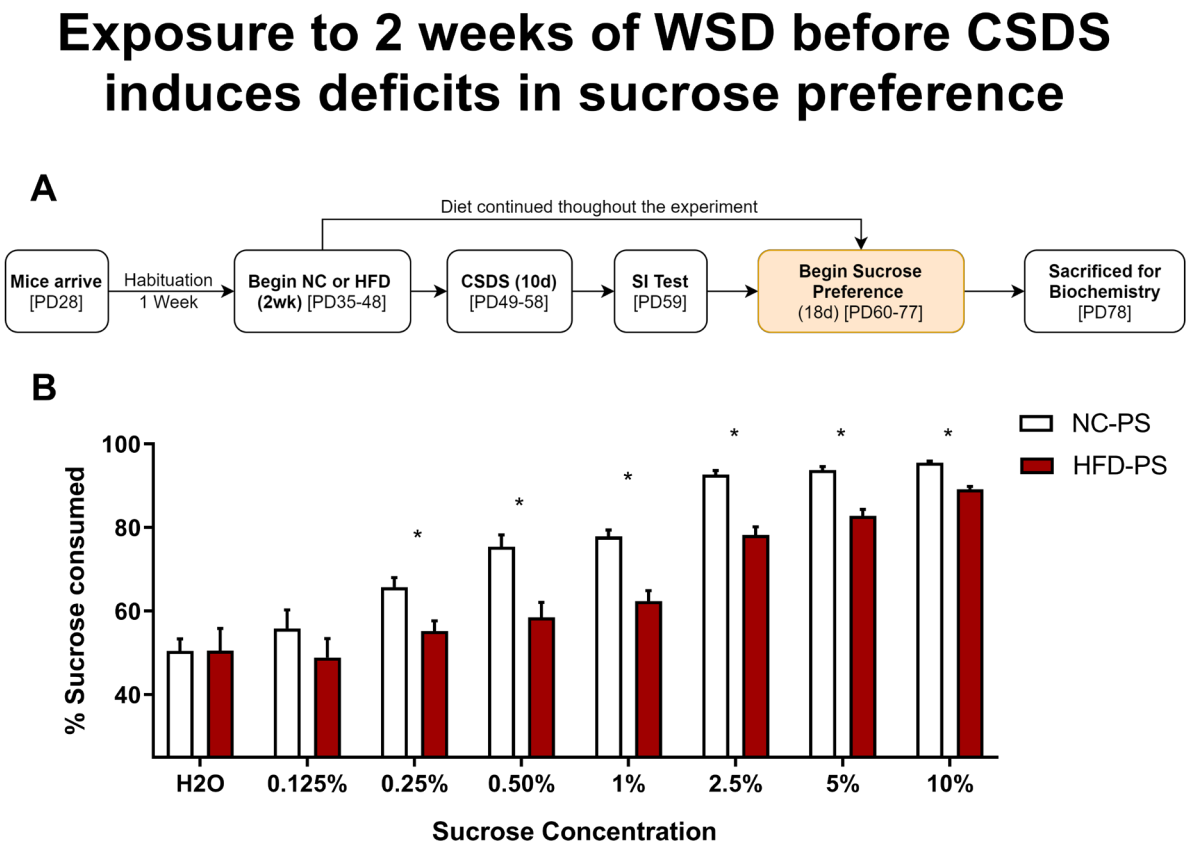
Figure 3: Exposure to 2 weeks of HFD before CSDS leads to increases in weight gain.



A) After the 1-week habituation period, the mice were moved into single-housing (PD35), with NC or HFD (n=12 per group) available ad libitum for 8 weeks (PD35-77). By the end of the first 2 weeks of diet, the mice were exposed to the chronic social defeat stress (CSDS; also referred to as physical stress; PS) depicted by the gray-shaded area, followed by the social interaction test

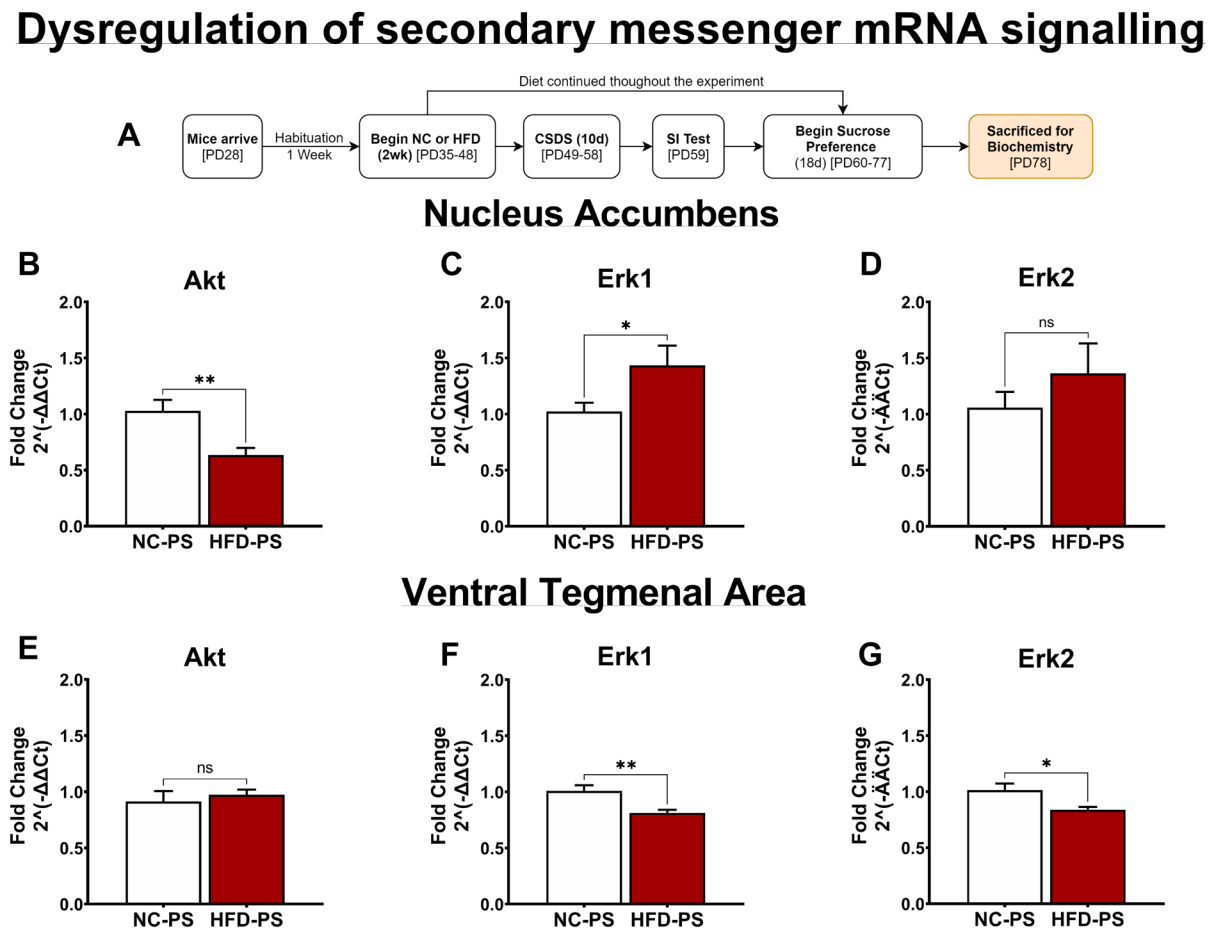
(SIT) 24 hours later to measure social avoidance. **B)** The mice in the HFD+PS condition exhibited increased body weight starting 1 week after the end of the defeat compared to the NC+PS-exposed mice, an effect that persisted for the remaining 4 weeks ($p<0.05$). **C)** The HFD+PS-exposed mice consumed significantly less food compared to NC+PS-exposed group for weeks 2-8 ($p<0.05$). **D)** There was a significant increase in adjusted caloric intake (i.e., calories consumed relative to body weight) in the HFD+PS- as compared to NC+PS-exposed mice in the first week ($p<0.05$), which was not present for the remaining 7 weeks. Data presented as kcal calories consumed/per gram of body weight. **E)** NC+PS- and HFD+PS-exposed mice exhibited significantly lower social interaction ratios compared to the CON group ($p<0.0001$). No differences in interaction ratio between the NC+PS and HFD+PS groups were detected. $^{*}p<0.05$: NC+PS and HFD+PS groups significantly different vs. CON. All data are shown as mean ± SEM.

Figure 4: Exposure to 2 weeks of WSD before CSDS induces deficits in sucrose preference in adulthood.



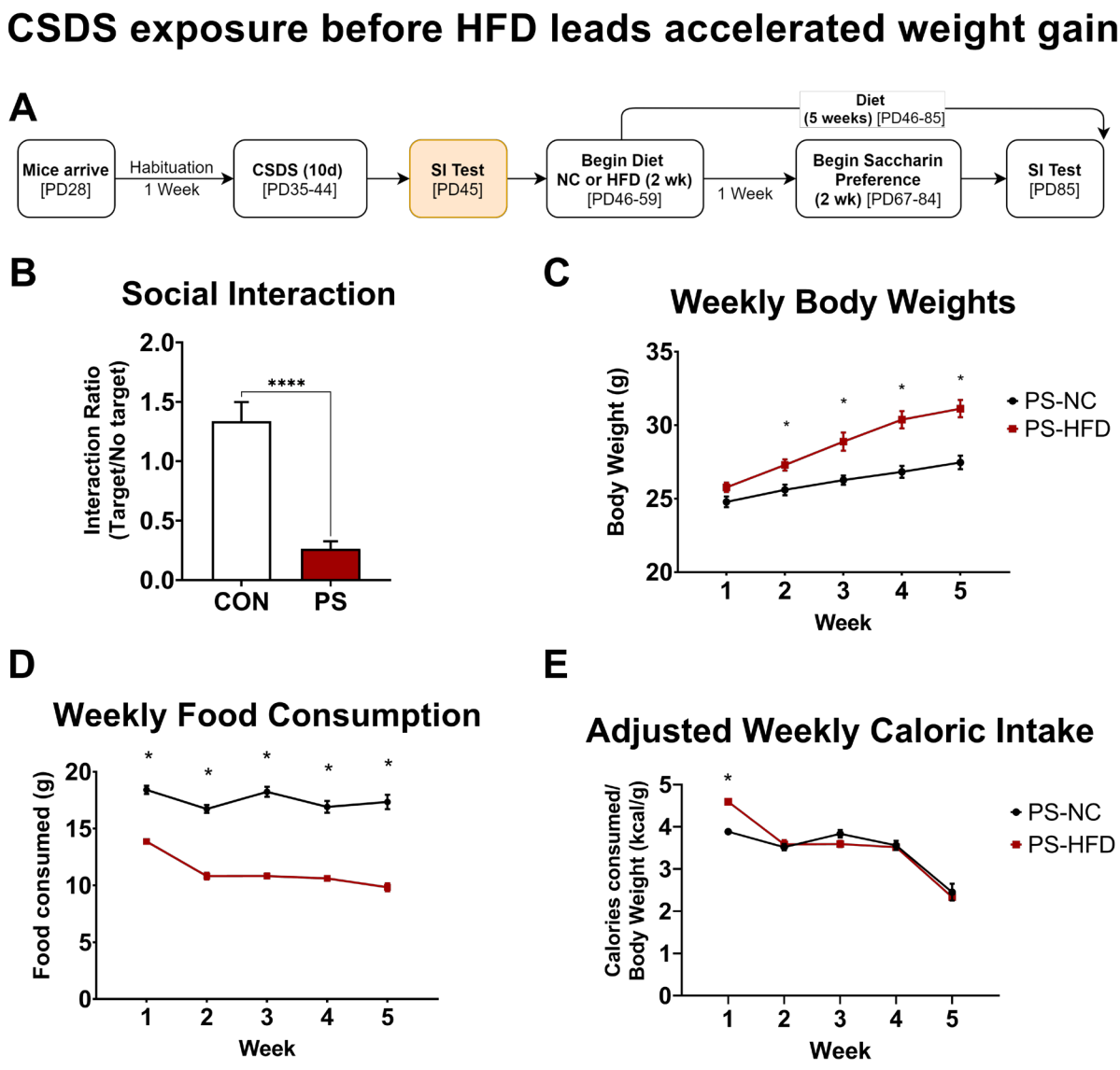
A) Two weeks after the SIT (PD59), the mice were exposed to the two-bottle sucrose preference test to assess for changes in reward sensitivity during adulthood (PD60-77). **B)** Mice in the HFD+PS condition consumed significantly less sucrose compared to the NC+PS-exposed mice at 0.25, 0.5, 1, 2.5, 5, and 10% concentrations (*p<0.05, respectively). Data are presented as percentage preference between the groups (mean ± SEM).

Figure 5: Exposure to 2 weeks of HFD before stress leads to dysregulation of secondary messenger systems within the mesolimbic system.



A) Brain punches from NAc and VTA were collected 24 hours after the last day of sucrose preference testing. (Figs. ***B-D***) t-test revealed a significant reduction in relative mRNA expression of *Akt* (*B*) and an increase in *Erk1* and *Erk2* within the NAc. (*C-D*). (Figs. ***E-G***) There were no significant differences in *Akt* levels between NC+PS- and HFD+PS-exposed mice (*E*). There was a significant reduction in the mRNA levels of both *Erk1* and EKR2 within the VTA in the HFD+PS-exposed mice (*F-G*) (*$p<0.05$, **$p<0.001$).

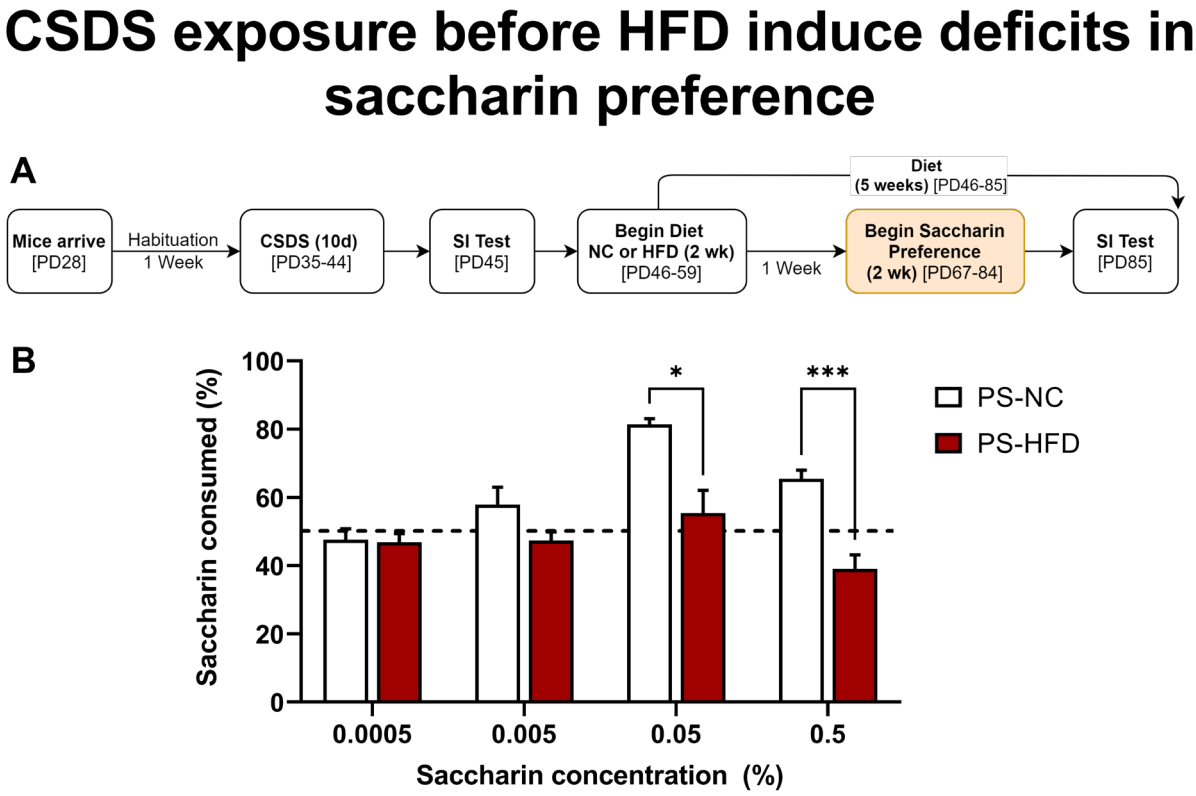
Figure 6: Effects of CSDS exposure followed by HFD on social interaction, weight gain, and caloric intake.



A) Timeline of experimental manipulation: after habituation, adolescent C576J mice were separated into control (CON; n=6) and chronic social defeat stress (CSDS/PS; n=24) groups. After exposure to CSDS, the mice were randomly separated into the two diet groups: NC or HFD exposure (n=12/group), followed by a SIT 24 hours after the last defeat to measure social avoidance. **B)** Mice in the CSDS condition showed a significantly lower social interaction ratio

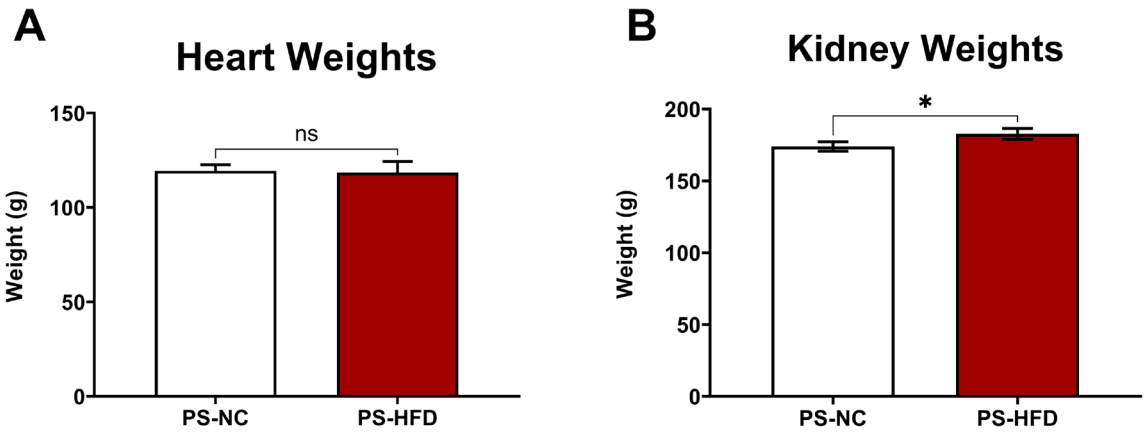
when compared to the CON-exposed mice ($p<0.05$). **C)** Mice in the PS+HFD condition exhibited rapid increases in body weight starting at week 2 compared to the PS+NC-exposed mice (n=12/group), and these changes persisted for the remaining 3 weeks. **D)** The PS+HFD-exposed mice consumed less food compared to the PS+NC-exposed group in weeks 1–5. **E)** There was a significant increase in adjusted caloric intake in the PS+HFD-exposed mice as compared to PS+NC-exposed group in the first week ($p<0.05$); this effect was not present for the remaining 4-weeks. *$p<0.05$: significantly different from the PS+NC-exposed group. All data are shown as mean ± SEM.

Figure 7: Effects of CSDS exposure followed by WSD on preference for saccharin.



A) A subgroup of adolescent mice was used to assess the effects of CSDS followed by HFD on reward sensitivity using the two-bottle preference test (n=8/group). **B)** Mice in the PS+HFD condition showed significantly reduced saccharin preference compared to the PS+NC-exposed mice at 0.05% and 0.5% saccharin concentrations. *p<0.05, ***p<0.001, respectively. Data are presented as percentage preference between the groups (mean ± SEM).

Figure 8: Effects of CSDS exposure followed by WSD on heart and kidney weight.



A) The same animals (n=8/group) from Figures 7-8 were sacrificed and their heart and kidneys harvested and weighed. There were no differences in the weight of the heart between the PS+NC- and the PS+HFD-exposed mice. **B)** the PS+HFD-exposed mice showed an increase in kidney weight compared to the PS+NC-exposed mice (*$p<0.05$).

CHAPTER III

EFFECTS OF EARLY-LIFE VICARIOUS SOCIAL DEFEAT STRESS AND WESTERN STYLE DIET ON BEHAVIORAL AND NEUROBIOLOGICAL PARAMETERS*

Introduction

The link between depression and obesity has been repeatedly demonstrated and is widely recognized as a highly prevalent and often life-threatening comorbidity (Luppino et al., 2010). The complexities of these individual conditions make the inquiries into their various interconnections and subsequent treatments exceedingly complex, thus modeling these conditions is of great importance. Unfortunately, most paradigms used in preclinical studies are unable to completely recapitulate these conditions in tandem due to several factors. For instance, a major drawback is that the majority of studies addressing depression and obesity issues have utilized adult rodents, overlooking adolescence, a sensitive developmental period in which there exists a pronounced vulnerability to palatable diets (Jacka et al., 2011) as well as stress (Eiland and Romeo, 2013). In addition, despite attempting to emulate the high saturated fat and refined carbohydrate content of the average, unhealthy foods found in the western hemisphere, these studies utilize diets that differ substantially in fat and carbohydrate content from the average western-style diet. Rodent studies use these very-high fat western-style diets (WSDs) in order to expedite physiological and behavioural deficits (Speakman, 2019); however, it may still take 6-20 weeks of consumption for deficits to emerge, resulting in rodents tested in late-adulthood and, at times, bordering geriatric

*Reprinted with permission from "Exposure to Vicarious Social Defeat Stress and Western-Style Diets During Adolescence Leads to Physiological Dysregulation, Decreases in Reward Sensitivity, and Reduced Antidepressant Efficacy in Adulthood Title of Article" by Sial, O.K., Gnecco, T., Cardona-Acosta, A.M., Vieregg, E., Cardoso, E.A., Parise, L.F., Guzman, C.B., 2021. Frontiers in Neuroscience, Volume 15, Page 930, Copyright 2021 by Frontiers.

age time-points (Moreno-Fernández et al., 2018). Together, these modeling limitations further amplify the need for working in models of early-life exposure in studying the link between diet and mood disorders. Furthermore, macronutrient composition of the diets has been understudied in this context, as high-fat foods have historically been overly implicated in the development of metabolic ailments like obesity, whereas the role of sugars has been neglected or downplayed (Kearns et al., 2018b). Adding to these complexities, stress has generally been ignored as a contributing factor in metabolic dysfunction leading to obesity or other diet-related disorders. Moreover, when stress paradigms are used, they are often not ethologically relevant and lack face and potentially internal validity. The use of pharmacological or non-natural stressors such as shock or restraint do not properly model the social stressors that humans experience, which can lead to both mood and metabolic dysregulation (Scott et al., 2012).

As briefly alluded to, adolescence is a period of enhanced vulnerability to stress, which can precipitate the emergence of major depressive disorder (MDD) and anxiety-related disorders (Eiland and Romeo, 2013), often causing life-long negative consequences (Kessler et al., 2007; Merikangas et al., 2010). Afflicted youth often adopt poor eating habits (Muñoz et al., 1997; O'Neil et al., 2014; Peeters, 2018), often deriving energy from foods high in fats and sugar (Reedy and Krebs-Smith, 2010). Not surprisingly, MDD is highly comorbid with obesity, which may be further precipitated by stress (Alastalo et al., 2013a; Loria et al., 2014; van Reedt Dortland et al., 2010; Vogelzangs et al., 2014; Weder et al., 2014). This bidirectional relationship between MDD and obesity is increasingly important as each entity may predict the onset of the other (van Reedt Dortland et al., 2010; Vogelzangs et al., 2014). The symptomatology of mood- and diet-related disorders often overlap and, though well-recognized, the mechanism(s) underlying this comorbidity are not well-understood (Mansur et al., 2015). Despite the prevalence of these specific

ailments, there are few translational research models that aim to understand how stress and diet interact simultaneously to precipitate symptoms like those found in various comorbid mood and diet-related disorders. Given the increasing incidence of both MDD (Mojtabai et al., 2016) and obesity in adolescents over the years (Marmorstein et al., 2014; Weiss et al., 2004), it is crucial to address this issue early in life to harbor better functionality in these individuals as they progress into adulthood.

Chronic stress, whether physical or emotional, during early-life can precipitate mood disorders such as MDD (Khan and Khan, 2017) and post-traumatic stress disorder (PTSD) (Kilpatrick et al., 2013). Patients suffering with PTSD are more likely to be susceptible to obesity and diabetes if also presenting with depressive symptoms (Hoerster et al., 2019). Emotional stress is a major contributor to the behavioral abnormalities that can also lead to physiological deficits (Escarfulleri et al., 2021). Furthermore, the prevalence of psychological/emotional stress in childhood is about four times that of physical stress (Vachon et al., 2015) and is highly predictive of the development of mental disorders later in life (Kessler et al., 2010). Given that solely witnessing a traumatic event is sufficient to precipitate anxious and depressive symptoms (Motta et al., 2004), it is also problematic that most animal models are unable to distinguish between physical and psychological stressors. The vicarious social defeat stress (VSDS) is a modified version of the chronic social defeat stress paradigm, an ethologically relevant and robust stressor that produces metabolic abnormalities (Chuang et al., 2010a, 2010b). The VSDS involves a C57BL/6J mouse solely "witnessing" a physical encounter for ~10 days, thus experiencing only psychological/emotional stress (Sial et al., 2016). The emotionally stressed mice exhibit increases in corticosterone levels along with depression- and anxiety-like behaviors similar to the physically stressed mice despite never having been engaged in a single bout of social defeat (Warren et al.,

2020). Metabolic abnormalities associated with chronic stress are unsurprising given the impact of stress on food intake, food preference, and visceral adiposity (Coccurello et al., 2018; Jayo et al., 1993; Scott et al., 2012). However, whether emotional stress exposure leads to similar physiological deficits is not known.

The consumption of poor diets is the largest modifiable risk factor of early death globally (Murray et al., 2016; Reisch, 2016). The western-style diet, also referred to as western-pattern diet, contains large amounts of saturated fats, sugars and refined grains (Shakersain et al., 2016). In addition, this diet has been strongly associated with long-term impairments such as dyslipidemia (Asadi et al., 2020), which often leads to cardiovascular disease(s), chronic inflammation (Neustadt, 2006), and cognitive decline (Shakersain et al., 2016). High carbohydrate diets and the consumption of sweetened beverages also pose a threat as they are known to increase serum triglycerides and reduce beneficial high-density lipoprotein (HDL) cholesterol (Coulston et al., 1983). Currently, there is a lack of studies that differentiate the effects of macronutrient content on various experimental endpoints. To address this gap, I exposed adolescent mice (PD35) to the VSDS paradigm in conjunction with WSDs (high in both fats and carbohydrates (HFD), or low fat and high carbohydrate (LFD)), to assess the complex interactions between stress, diet, and reward regulation. The aim of the studies presented here is to characterize deficits in weight gain, social interaction, reward, and responses to antidepressant intervention. I hypothesize that exposure to emotional stress will accelerate the detrimental physiological and behavioral effects induced by WSDs, specifically a diet high in both fats and carbohydrates (HFD).

Methods

Animals

Male C57BL/6J mice (C57; Jackson Labs, Bar Harbor, Maine), postnatal day (PD) 28 at time of arrival, and CD-1 retired breeders (Charles Rivers, North Carolina) were housed at 23°C, in clear polypropylene boxes, on a twelve-hour light/dark cycle (lights on at 7AM, lights off at 7PM) and habituated for 7 days. The C57 mice were group-housed during habituation and moved to single housing at PD35, the start of experimental manipulations, while the CD-1 mice were singly housed from arrival. All experimental procedures were conducted in strict compliance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003) and approved by Texas A&M University's Animal Care and Use Committee.

Stress

Vicarious social defeat stress (VSDS) was performed as described previously (Sial et al., 2016). Adolescent mice were randomly assigned into control (CON), emotional (ES), or physical stress (PS) conditions and exposed to 10 days of VSDS lasting 10 minutes each. Briefly, the home cage of the CD-1 mouse was separated into two compartments by a perforated clear Plexiglas divider. During the daily stress sessions, the intruder mouse (PS) was placed into the territorialized home-compartment of a CD-1 and subsequently physically defeated. At the same time, a different experimental mouse (ES) in the adjacent compartment witnessed the defeat bout, mimicking a form of emotional social stress. At the end of the defeat session, the ES-exposed mouse was moved to stay overnight in an adjacent cage that was not used that day for defeats. The PS-exposed mouse

was placed overnight in the compartment adjacent to the CD-1 that socially defeated it, for overnight sensory exposure. To minimize physical injury, the daily sessions were terminated when the intruder mouse adopted a persistent submissive posture, or if the CD-1 displayed excessive physical aggression. Mice in the CON condition were moved to new compartments daily with no contact with a CD-1.

Diet

Mice were further randomized to receive either standard normal chow (NC; Teklad Rodent Diet; 8604; fat content 14% kcal, carbohydrate content 54% kcal from starches; 3.0 kcal/g), or a diet high in both fats and carbohydrates (HFD; Research Diets; D12451; fat content 45% kcal from lard, carbohydrate content 35% kcal from sucrose; 4.73 kcal/g) ad libitum. A separate diet, low in fat but containing the same amount of added sucrose as the HFD was also utilized (LFD; Research Diets; D12450K; fat content 10% kcal from lard, carbohydrate content 70% kcal from sucrose; 3.82 kcal/g). Information about the diets can be found in Table 2. All food was placed in metal food hoppers and weighed daily. Caloric intake was measured by multiplying the daily consumption of food (g) by the caloric content (kcal/g) of the diet. The adjusted caloric intake is a normalized value that represents the number of calories consumed relative to body weight (g) of the mouse ((kcal/g of diet * grams of diet consumed)/body weight).

Social Interaction Test

The social interaction test (SIT), a behavioral assay assessing social avoidance, is used as the primary behavioral outcome measure after exposure to the VSDS paradigm. The SIT was performed 24 hours after the last defeat session or after diet or fluoxetine exposure. Briefly, the

SIT is composed of two, 2.5 minute-sessions. In the first session, a mouse is allowed to explore an open field arena (40 cm × 40 cm) containing an empty wire mesh cage (i.e., no social target present). For the second session, the mouse is then removed, and a novel CD-1 mouse is placed into the wire mesh cage (i.e., social target present). The test mouse is returned to the arena and the amount of time spent in the "interaction zone" (8 cm wide corridor surrounding the wire mesh cage), as well as the time spent in the corners farthest from the social target is measured. The VSDS-defeated mice explore the interaction zone significantly less when a target mouse is present and spend more time in the corners. Social interaction is calculated as a ratio of time spent in the interaction zone, with and without the target present (time in interaction zone with target/time in interaction zone without target). Interaction ratios below 1.0 indicate social avoidance and susceptibility to stress.

Saccharin Preference Test

The saccharin preference test is a two-bottle choice paradigm in which mice are given unrestricted access to both water and saccharin (Sigma-Aldrich: 240931). This paradigm has been used extensively to assess the effects of stress-induced anhedonia (Eagle et al., 2016; Guan et al., 2015). Testing began 48 hours after the last defeat session. Mice were habituated to two bottles for two days, and every 24 hours the bottles were weighed, and their positions rotated (left to right and vice versa) to account for any side preferences. Mice were exposed to water and saccharin (0.05%, 0.1%, 0.5%) for 48 hours per concentration. The preference for saccharin over water [e.g. saccharin/(saccharin + water)] was used as a measure of sensitivity to reward.

Fluoxetine Reversal

To determine potential changes in antidepressant efficacy after VSDS+WSD exposure, mice were given access to fluoxetine for 21 days. Fluoxetine hydrochloride (FLX; TCI America: F0750) was dissolved in the drinking water (80 mg/L) and made available ad libitum. The FLX dose was selected to achieve plasma levels close to 10 mg/kg (Dincheva et al., 2017). This treatment approach was used to avoid potential stress-induced weight loss and/or tissue damage due to injection (Perrone et al., 2004). Bottles were covered in aluminum foil to prevent photodegradation. The FLX solution was replaced every 48 hours to ensure purity and accurate concentration.

Statistical analyses

Data were analyzed using SPSS (version 26) and GraphPad Prism (version 9) software. Changes in body weight, food consumption, and adjusted caloric intake were compared using multivariate analysis of variance (MANOVA), followed by Tukey HSD post-hoc tests with stress (CON, ES, PS), diet (NC, LFD, HFD), and time (averaged 7 days across week: repeated measure) as sources of variance. When appropriate, two-way ANOVAs or Student's t-tests were used to determine statistical significance of pre-planned comparisons. Data are expressed as the mean ± SEM, with statistical significance set at $p<0.05$.

Results

Effects of VSDS exposure followed by HFD on body weight, food consumption, caloric intake, and social interaction.

Experimental Design. After a week habituation period, adolescent mice were randomly assigned into CON and VSDS (ES or PS) conditions (n=12/group) and exposed to VSDS for 10 days (PD35-44), followed by a SIT 24 hours after the final defeat session (see shaded square, PD45). Following VSDS exposure, the mice were randomized further into either the NC or HFD conditions to assess the effects of VSDS+HFD on body weight, food consumption, and adjusted caloric intake (Fig. 9*A*).

Social Interaction Before Diet. To verify the efficacy of VSDS exposure, mice were assessed for social interaction as measured in the SIT. Interaction ratios were calculated as described. A two-way ANOVA revealed a significant main effect of stress ($F_{(2,22)}$= 12.90; p<0.0002). Post-hoc analysis showed that the interaction ratio of both ES- and PS-exposed mice were significantly lower (i.e., social avoidance) than the mice in the CON condition, indicating a successful defeat (Fig. 9*B*). Although the PS-exposed mice had lower SI scores, the magnitude of social avoidance did not significantly differ when compared to the ES-exposed group (p>0.05).

Body weight. Measurements of body weight were obtained daily, starting across the defeats, throughout the 4 weeks of diet exposure, and averaged weekly (Fig. 9*C*). A 3 (stress) x 2 (diet) x 5 (time: weekly body weight as repeated measured variable) MANOVA revealed a significant three-way interaction between stress, diet, and time ($F_{(8,54)}$= 2.23, p<0.05; Wilks' Λ= 0.565). Post-hoc analyses revealed that at the final week of food consumption, the ES+HFD- and PS+HFD-exposed mice were significantly heavier than the CON+NC-exposed group (p<0.0025 and p<0.0007, respectively). Surprisingly, only the ES+HFD-exposed group differed significantly

from the CON+HFD-exposed group ($p<0.01$). No changes in body weight between the ES+HFD- and PS+HFD-exposed groups were detected ($p>0.05$).

Food consumption. The amount of food consumption was measured daily across the 4 weeks of diet and averaged weekly (Fig. 9*D*). A MANOVA showed that food consumption was influenced by time ($F_{(3,28)}= 54.07$; $p<0.05$; Wilks' $\Lambda= 0.147$) and diet ($F_{(3,28)}= 9.99$; $p<0.05$; Wilks' $\Lambda= 0.483$), but not stress ($F_{(6,56)}= 2.17$; $p>0.05$; Wilks' $\Lambda= 0.658$). Post-hoc analysis further revealed that from weeks 2-to-4 of diet exposure, the mice in the NC condition consumed more food than the mice in the HFD condition ($p<0.05$).

Adjusted Caloric Intake. Caloric intake was measured and converted to adjusted caloric intake and averaged across the 4 weeks of diet exposure (Fig. 9*E*). A MANOVA revealed significant changes in adjusted caloric intake as a factor of time ($F_{(3,28)}= 89.49$; $p<0.05$; Wilks' $\Lambda= 0.111$) and diet ($F_{(3,28)}= 17.93$; $p<0.05$; Wilks' $\Lambda= 0.506$), but not stress ($F_{(6,58)}= 1.37$; $p>0.05$; Wilks' $\Lambda= 0.774$). Post-hoc analysis detected significant differences in the first week between the HFD- and NC-exposed mice without differences seen between the groups during the final week.

Long term social interaction after VSDS and HFD exposure.

Social Interaction After Diet. To evaluate whether 4 weeks of HFD after VSDS would influence social interaction, mice underwent a SIT 24 hours after the last day of diet exposure (Fig. 10*A*; shaded square, PD74). A two-way ANOVA indicated only a significant effect of stress ($F_{(2,30)}= 35.91$; $p<0.0001$; Fig. 10*B*). Post-hoc analyses showed that mice in both the ES+HFD ($p<0.02$) and PS+HFD ($p<0.0002$) conditions retained their social avoidance phenotype, as they were significantly different from the CON+HFD-exposed group. More specifically, mice in the PS condition retained their defeated phenotype, as the PS+NC- and PS+HFD-exposed groups

showed significantly more social avoidance when compared to the CON+NC control mice (p<0.01, respectively). Although the mice in the ES+NC condition showed a moderate increase in social interaction from baseline after 1 month of diet exposure, the ES+HFD-exposed mice still showed a significant reduction in interaction (p<0.05) when compared to the mice in both CON+NC and CON+HFD conditions.

Effects of VSDS exposure followed by HFD on saccharin preference.

To evaluate the effects of VSDS+HFD exposure on reward sensitivity, mice were assessed for preference to a sweetened water solution for 12 days (n=6/group; PD75-86) using a two-bottle preference test, 24 hours after the last SIT (Fig. 11*A*). To this end, a two-bottle saccharin preference test with varying saccharin concentrations was performed (Fig. 11*B*). A MANOVA revealed differences in preference for the sweetened solution as a factor of saccharin concentration ($F_{(3,28)}$= 122.39; p<0.05; Wilks' Λ= 0.071) and diet ($F_{(3,28)}$= 7.11; p<0.05; Wilks' Λ= 0.568). Post-hoc analyses revealed that there were no significant differences between the groups at the 0.05% and 0.1% concentrations. However, both the ES+HFD- and PS+HFD-exposed mice showed a significant reduction in preference for saccharin at the 0.5% when compared to the CON+NC-exposed group (p<0.05). Furthermore, only the PS+HFD-exposed group was significantly different when compared to the CON+HFD-exposed mice (p<0.05). Inset graph shows the raw water consumption during the water baseline. A two-way ANOVA showed that the CON+HFD- and the PS+HFD-exposed mice drank less water than their respective NC-exposed counterparts (p<0.05).

Effects of VSDS exposure followed by LFD or HFD on body weight, food consumption, caloric intake, and social interaction.

To further elucidate whether the observed effects thus far were due to HFD consumption, a separate control diet, similar in carbohydrate content but lower in fat (LFD), was introduced.

Experimental Design. After a week of habituation, adolescent male C57 mice were exposed to 10 days of VSDS (n=6/group), followed by 4 weeks of either NC, LFD or HFD, and then assessed in the SIT (Fig. 12*A*). After the SIT, these mice were exposed to 21 days of fluoxetine in their drinking water, followed by another SIT (PD95), results which are shown in Fig. 13*B*.

Body Weight: A MANOVA detected significant changes in body weight as a result of stress, diet, and time interactions ($F_{(8,54)}$= 2.21, p<0.05; Wilks' Λ=0.568; Fig. 12*B*). Post-hoc analyses show that ES- and PS-exposed mice in the HFD condition gained weight more rapidly and were significantly heavier when compared to the LFD-exposed mice, regardless of stress condition during the final week of diet exposure (p<0.05; inset graph). Additionally, the ES+HFD-exposed mice were significantly heavier than the CON+HFD group (p<0.05; inset graph).

Food Consumption: Changes in food intake were apparent as a factor of both time ($F_{(8,54)}$= 2.21, p<0.05; Wilks' Λ= 0.323) and diet ($F_{(8,54)}$= 2.21, p<0.05; Wilks' Λ= 0.313; Fig. 12*C*). Post-hoc analysis revealed that the mice in the LFD condition consumed more food than the HFD-exposed mice regardless of stress condition, likely a result of the lower caloric value of the LFD.

Adjusted Food Intake. Three-way MANOVA showed that differences in adjusted caloric intake were a factor of time ($F_{(3,28)}$= 151.89, p<0.05; Wilks' Λ= 0.152) and diet ($F_{(3,28)}$= 32.39, p<0.05; Wilks' Λ= 0.224; Fig. 12*D*). No significant differences were detected between the groups during the final week of diet exposure (p>0.05).

Social Interaction. After 4 weeks of diet exposure, the mice were tested for SI. A two-way ANOVA detected significant changes in SI as a factor of stress ($F_{(2,30)}$= 26.78, p<0.0001; Fig. 12*E*) but not diet exposure ($F_{(1,30)}$= 0.155; p>0.05). Post-hoc analysis revealed that VSDS-exposed mice showed significant reduction in SI regardless of stress (ES or PS) or diet (LFD or HFD) conditions when compared to their respective CON counterparts (p<0.05).

Effects of fluoxetine treatment on VSDS+LFD- or HFD-induced social avoidance.

Mice from the same cohort reported in Figure 13 were given fluoxetine (FLX; 80 mg/L) for 21 days in the drinking water after exposure to VSDS and either NC, LFD, or HFD (Fig. 13*A*). A two-way ANOVA revealed that differences in antidepressant efficacy were a factor of both stress ($F_{(2,36)}$= 22.39; p<0.0001; Fig. 13*B*) and diet ($F_{(2,36)}$= 5.525; p<0.0081). Post-hoc analysis showed that FLX treatment rescued the social interaction ratio of the mice in the ES+NC and PS+NC conditions. Interestingly, the LFD- and HFD-exposed mice showed an attenuated response to FLX regardless of stress condition when compared the CON+NC-exposed mice. More specifically, both the ES+LFD- and PS+LFD- as well as the ES+HFD- and PS+HFD-exposed mice showed significantly lower interaction ratio when compared to their respective CON+LFD (p<0.05), and CON-HFD (p<0.05) groups. These findings indicate that WSD attenuates the antidepressant efficacy of FLX.

Discussion

The public health implications of the comorbidity between depression and obesity are a major cause for concern; thus, establishing animal models that can mimic this condition is of the utmost importance. Though studies using stress and diet have independently begun to elucidate some of the mechanisms that may underlie the phenomenon, there are very few studies utilizing both early-life stress and western style diets (WSDs) to understand the synergistic detrimental effects. The most common form of abuse experienced by adolescents is emotional or psychological abuse, which has been shown to be just as detrimental as physical abuse (Spinazzola et al., 2014). To fully understand whether psychological stress would have similar effects to physical stress within the context of WSDs, I utilized the vicarious social defeat stress (VSDS) paradigm, an ethologically relevant and robust stressor that allows for the uncoupling of emotional stress (ES) from physical stress (PS). This paradigm has demonstrated that simply witnessing repeated physical defeats evokes many of the same behavioral deficits (e.g., reduced social interaction, lower latency to immobility, anhedonia) as those of direct PS exposure (Warren et al., 2011). Following 10 days of VSDS exposure, adolescent mice were exposed to 4 weeks of either normal chow (NC) or high fat diet (HFD). Here, I found that both ES- and PS-exposed mice in the HFD condition rapidly gained weight (within 1 week after VSDS exposure) when compared to non-stressed control (CON) and the VSDS-exposed mice in the NC condition at the end of the 4 weeks. Interestingly, ES+HFD-, but not the PS+HFD- were significantly heavier than the CON+HFD-exposed mice. The similarities in body weight between the PS+HFD- and the CON+HFD-exposed mice have been demonstrated in adult studies where the mice in the PS+HFD condition weigh more than the mice in the CON+NC but not the CON+HFD condition (Chuang et al., 2010a). Curiously, the rapid weight gain presented here in adolescent mice is independent of food or caloric

intake, as the HFD-exposed mice consumed fewer grams of the calorically-dense diet, regardless of stress condition; and show no differences in adjusted caloric intake, despite the novelty-induced hyperphagia observed following single-housing and introduction to the novel diet. Exposure to stress has previously been shown to decrease food intake (E. Morley et al., 1983) and decrease weight gain (Ip et al., 2019; Warren et al., 2013), thus the nominal change in caloric intake in adolescents is intriguing given their rapid increase in body weight. The mechanism(s) underlying this phenomenon in adolescents is unknown. In adult mice, increases in caloric intake of palatable diets is observed when first exposed to social stress (Coccurello et al., 2018; Hassan et al., 2019). Given that PS exposure can cause changes in lipid metabolism and energy distribution (Chuang et al., 2010a), it is possible that shifts in metabolic processing are occurring more drastically in adolescence after stress exposure, therefore resulting in rapid weight gain despite consuming the same amount of calories as the mice in the NC condition. Further studies are needed to explore this hypothesis and determine whether altered cellular respiration pathways take place during adolescence to preferentially store and metabolize different sources of macronutrients.

Social avoidance is an integral symptom of depression and can be quantified in rodents using the social interaction test (SIT), a measure of stress reactivity and depressive/anxiety-like behaviors (Overstreet, 2012). An initial baseline measurement of avoidance was taken 24 hours after the last VSDS exposure. As expected, both the ES- and PS-exposed mice showed significant reduction in social interaction compared to the mice in the CON condition. To evaluate whether HFD exposure influenced social interaction further, mice were retested in the SIT 4 weeks after diet exposure. Interestingly, the PS+NC- and the PS+HFD-exposed mice retained their socially avoidant phenotype after diet consumption. Some studies have yielded inconsistent findings,

showing that exposure to HFD before or during stress can buffer against the negative effects of PS (Finger et al., 2011; MacKay et al., 2017). The ES+HFD-exposed mice maintained comparable avoidant phenotypes as observed before diet exposure. These data show that 4 weeks of HFD has no effect on social interaction in either the ES- or PS-exposed mice. Additionally, previous studies confirm that HFD-exposure does not ameliorate the effects of stress and can in fact induce depressive-like behaviors (Pan et al., 2019). Given the low interaction ratio of PS-exposed mice, it is likely that any effects of HFD-exposure on social behavior would be unseen due to the floor effect.

The effect of stress and HFD on reward reactivity to saccharin, an artificial sweetener devoid of calories, was assessed using a two–bottle preference test, where the readout is given as a percentage of saccharin consumed over plain water. I report that mice exposed to either ES or PS showed decreased preference to 0.5% saccharin, a behavioral phenotype often associated with anhedonia (i.e., lack of pleasure), when compared to the non-stressed CON+NC-exposed mice. The mice in the VSDS+HFD condition demonstrated less than 50% preference for saccharin, which could be interpreted as an aversion to the 0.5% saccharin concentration. There was a trend toward reduced consumption in VSDS+HFD-exposed mice at the 0.1% concentration, however, this experiment may need to be repeated with a larger cohort to confirm these findings. Given the essential role of the mesolimbic dopamine (DA) system in food consumption and reward (Volkow et al., 2017; Wise, 2013), it is conceivable that stress and HFD exposure compromise this pathway. The brain's reward pathway, which includes the nucleus accumbens (NAc) and its dopaminergic input from the ventral tegmental area (VTA), are involved in regulating motivated behavior, along with responses to drugs of abuse and natural rewards (Bolaños and Nestler, 2004; Di Chiara and

North, 1992; Kelley and Berridge, 2002; Wallace et al., 2008). Experimental evidence indicates that exposure to sweet solutions such as sucrose are rewarding (Datla et al., 2002; Hajnal and Norgren, 2001) in that they activate the mesolimbic dopaminergic system, resulting in increased DA release in the NAc, whereas lesions to this reward pathway block sucrose preference (Shimura et al., 2002). Reward dysregulation is a common phenotype that often emerges after early-life chronic exposure to palatable substances (Sato et al., 1991; Teegarden et al., 2009), drugs of abuse (Iñiguez et al., 2009; Naneix et al., 2017), or stress (Iñiguez et al., 2010a; Parise et al., 2013). Chronic consumption of diets high in fats and carbohydrates have also been shown to induce long-term changes in DA neurotransmission (Naneix et al., 2018; Teegarden et al., 2009), thus influencing reward sensitivity. Exposure to VSDS decreases sucrose preference in both adults (Krishnan et al., 2007; Warren et al., 2013) and adolescent mice (Iñiguez et al., 2014b) and creates deficits associated with reduced reward sensitivity and anhedonia-like behavior in rodents (Eagle et al., 2016). My results agree with these findings and further extend our current understanding to demonstrate that early-life stress in conjunction with HFD exposure induces deficits in reward sensitivity compared to stress alone. It should be noted that the CON+HFD-exposed mice consumed less water at baseline than the mice in the CON+NC condition, a phenomenon also observed in the PS-exposed mice, while the ES+HFD-exposed mice showed a non-significant decrease in water intake. The mechanism(s) underlying this effect is unknown. Though HFD influenced fluid intake, these effects are controlled for by taking a percentage of saccharin consumed over total intake.

Although there is substantial evidence delineating the long-term detriments induced by western-style diet consumption (Zemdegs et al., 2016), the specific macronutrient composition

that may directly or indirectly cause these deficits has not been clearly identified. Traditionally, high-fat diets have been blamed for diet-related disorders such as obesity and metabolic syndrome (MetS), though recently, the role of carbohydrates has come under scrutiny for their involvement in these comorbidities (Kearns et al., 2018a). To assess how HFD exposure differed from a diet that had low fat, but the same carbohydrate content (LFD; 35 %kcal from sucrose; see Table 2), adolescent mice were exposed to VSDS before 4 weeks of either HFD or LFD exposure. Though the mice exposed to either diet consumed the same amounts of adjusted calories, only the ES+HFD- and PS+HFD-exposed mice rapidly gained weight as demonstrated previously (see Chapter II, Fig. 6*B*). This indicates that high carbohydrates were not enough to induce rapid weight gain in the stress-exposed mice, and a combination of high fat and high carbohydrate content was necessary to induce these physiological effects. In addition, there was no significant difference in social interaction between the HFD- and LFD-exposed mice regardless of stress condition, as they retained their avoidant phenotype. Given that chronic antidepressant treatment can reverse social avoidance after VSDS exposure (Warren et al., 2013), I tested whether 21-day exposure to the selective serotonin reuptake inhibitor fluoxetine (FLX; 80 mg/L in the drinking water) could reverse the social interaction deficits observed. When tested in the SIT 24 hours after the last day of FLX consumption, the ES+NC and PS+NC mice in the FLX condition were not statistically different from the mice in the CON condition. Conversely, exposure to HFD or LFD blocked FLX's ability to reverse the socially avoidant behavior regardless of stress condition. I thus show here that although mice exposed to stress do not gain weight as rapidly when consuming LFD, they share similar behavioral deficits as the VSDS+HFD-exposed mice. Resistance to FLX treatment has previously been shown in adult mice subjected to two separate 7-week periods of unpredictable chronic mild stress while consuming a HFD (Isingrini et al., 2010). The

mechanism(s) mediating this phenomenon are not known. Although speculative, reduced FLX efficacy may be due to over-sequestration of the lipophilic FLX into adipose stores (Shekar et al., 2012), or due to WSD-induced inflammation (Wu et al., 2018). The latter seems to be supported by studies showing that anti-inflammatory agents such as acetylsalicylic acid, when given with FLX, increase FLX's antidepressant efficacy in animal models (Wang et al., 2011; Yang et al., 2014; Zdanowicz et al., 2017). There exists a strong association between MetS and mood disorders, especially depression (Chan et al., 2019; Hiles et al., 2016; Penninx and Lange, 2018). Depending on the remission criteria utilized, the rate of treatment-resistant depression (TRD) ranges between 35% and 60% (Fekadu et al., 2009; Nemeroff, 2007), with TRD being associated with higher rates of cardiovascular mortality (Carney and Freedland, 2009). Interestingly, diet-related disorders (e.g. MetS) contribute significantly to the sustained chronicity of depression and low rates of antidepressant efficacy (Chokka et al., 2006; van Reedt Dortland et al., 2010; Vogelzangs et al., 2011, 2014). These findings therefore indicate that diet plays a pivotal role in antidepressant efficacy and highlight the importance of considering diet when prescribing antidepressants.

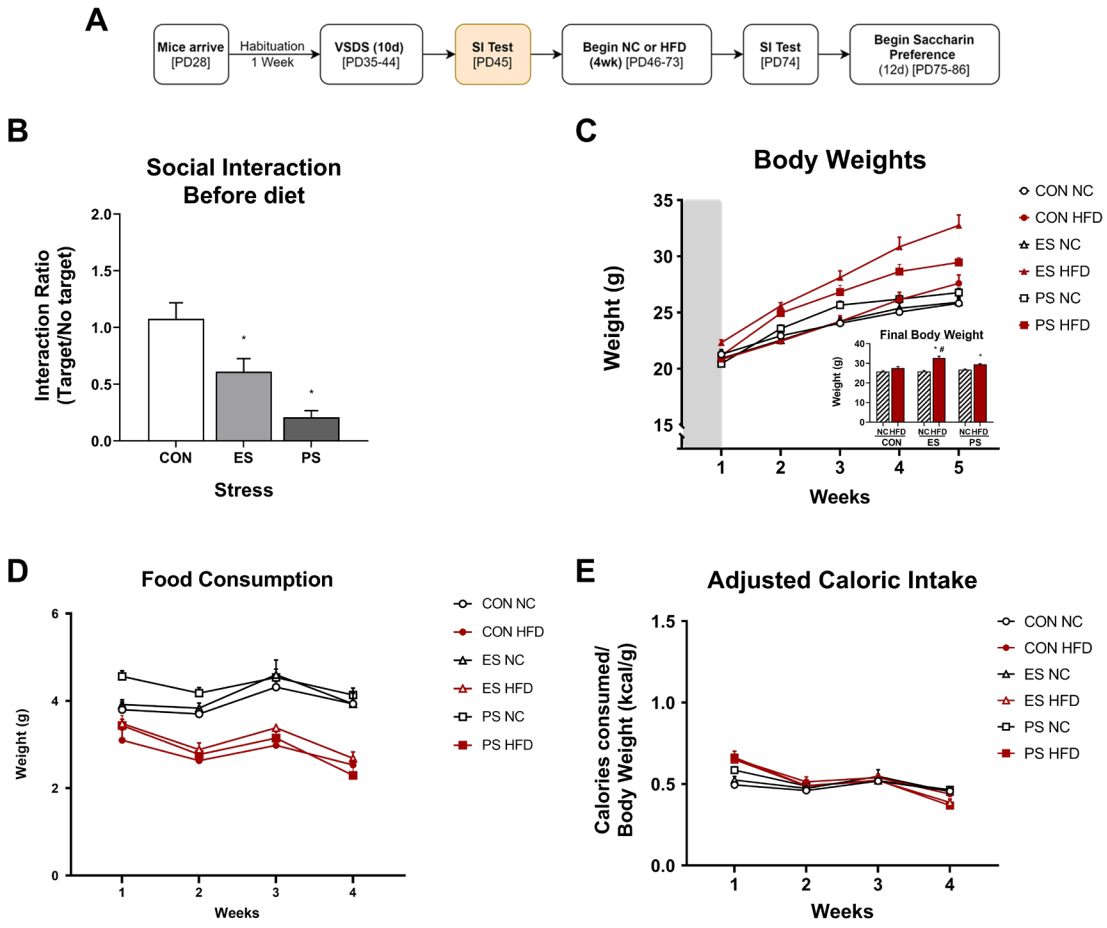
Some limitations of this study include the use of only male mice and the lack of a micronutrient and protein-controlled chow. There is evidence that women are at an increased risk of developing obesity after a stressful event and are more likely to suffer from depression compared to males (Mannan et al., 2016). The stress paradigm utilized here is not amenable to the use of female mice, as there is no validated method of successfully encouraging males to aggressively charge female mice. Although some approaches have involved the use of more aggressive strains of female mice, optogenetic manipulations in transgenic mice, and male pheromones to incite males to show

aggression toward a female (Harris et al., 2018; Newman et al., 2019; Takahashi et al., 2017), these new models have not been yet been fully characterized and validated. Another limitation is the normal chow used in this study is what is commonly given to rodents in many laboratories across the country, though using a matched-control chow would have excluded the possibility of off-target effects. Furthermore, studies assessing physiological measures such as corticosterone, insulin, and inflammatory markers would be critical in validating the paradigm of adolescent metabolic-mood syndrome.

Together, these findings indicate that either psychological or physical stress combined with a high-fat/high-carbohydrate during adolescence can induce in a rapid obesogenic phenotype and reward-related deficits (i.e., anhedonia) that may lead to the development of maladaptive behaviors and negative health outcomes in adulthood. We describe a paradigm that can elicit a rapid obesogenic-like phenotype, along with deficits in reward and antidepressant response in adulthood. Elucidating the relationship between MDD and MetS may uncover crucial implications for adolescent health and sociocultural patterns of behavior for future adult functioning. Further, it is crucial to understand the neurobiological interactions between mood and metabolic disorders, as environmental insults such as social stress and unhealthy diet can cause long-lasting behavioral and physiological deficits that persist into adulthood (Lobstein et al., 2004).

Figures

Figure 9: Effects of VSDS exposure prior to 4 weeks of HFD on social interaction, body weight, food, and caloric intake.

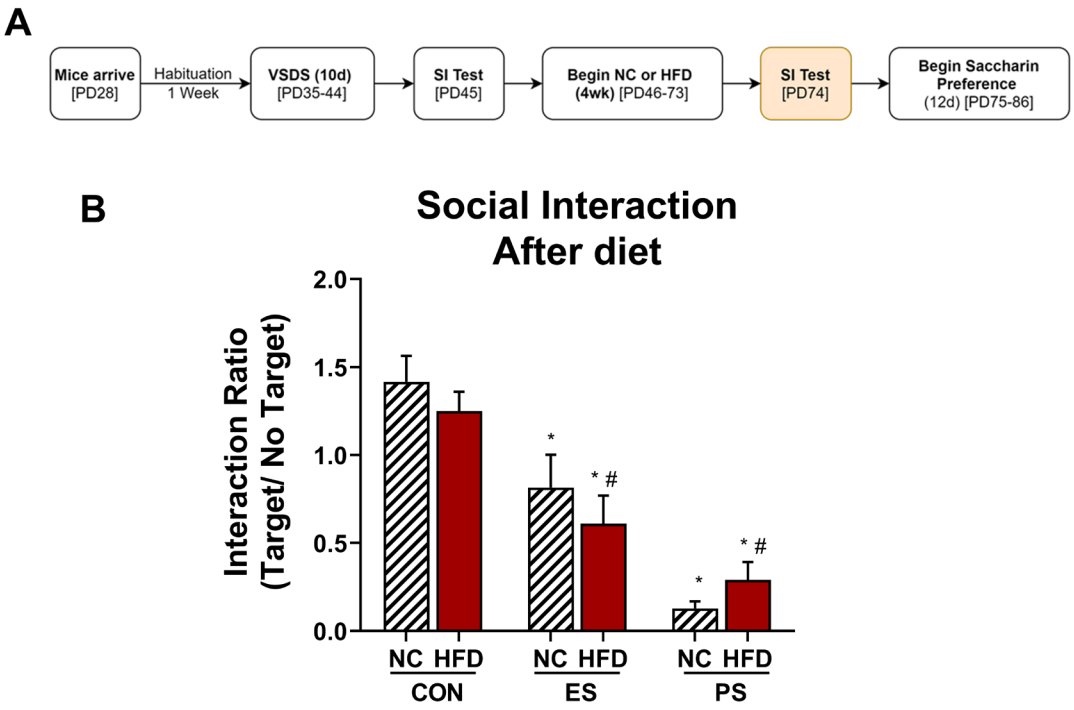


A) Adolescent mice were exposed to the vicarious social defeat stress (VSDS) paradigm and subsequently tested for social interaction. After the SIT, the mice were randomly assigned to the normal chow (NC) or the high fat diet (HFD) conditions, followed by an additional SIT on PD74. **B)** Mice in both the emotional (ES) (n=12, $p<0.001$) and physical (PS) stress (n=12, $p<0.05$) conditions showed a significant reduction in social interaction when compared to the CON-exposed mice (n=12) with a main effect of stress ($p<0.001$). **C)** Mice that received HFD in both

ES and PS conditions gained weight rapidly and were significantly heavier than the mice in the CON-NC condition during the final week of diet exposure (post-hoc: $p<0.05$) with a significant interaction between stress, diet, and time (separated n=6/group, $p<0.05$). **(*D*)** HFD-exposed mice consumed less grams of the calorically dense diet compared to the NC-exposed mice during weeks 2-4 ($p<0.05$), showing that the feeding behaviour of the mice was homeostatically regulated. **E)** No differences in adjusted caloric intake between any of the groups in the final week of diet exposure were noted (post-hoc, $p>0.05$), though there were significant differences in the first week (main effect of time and stress, $p<0.05$

$^{*}p<0.05$: significantly different than the CON-NC; $^{\#}p<0.05$: significantly different than CON-HFD. All data are expressed as the mean ± SEM. Reprinted with permission from Sial et al., 2021.

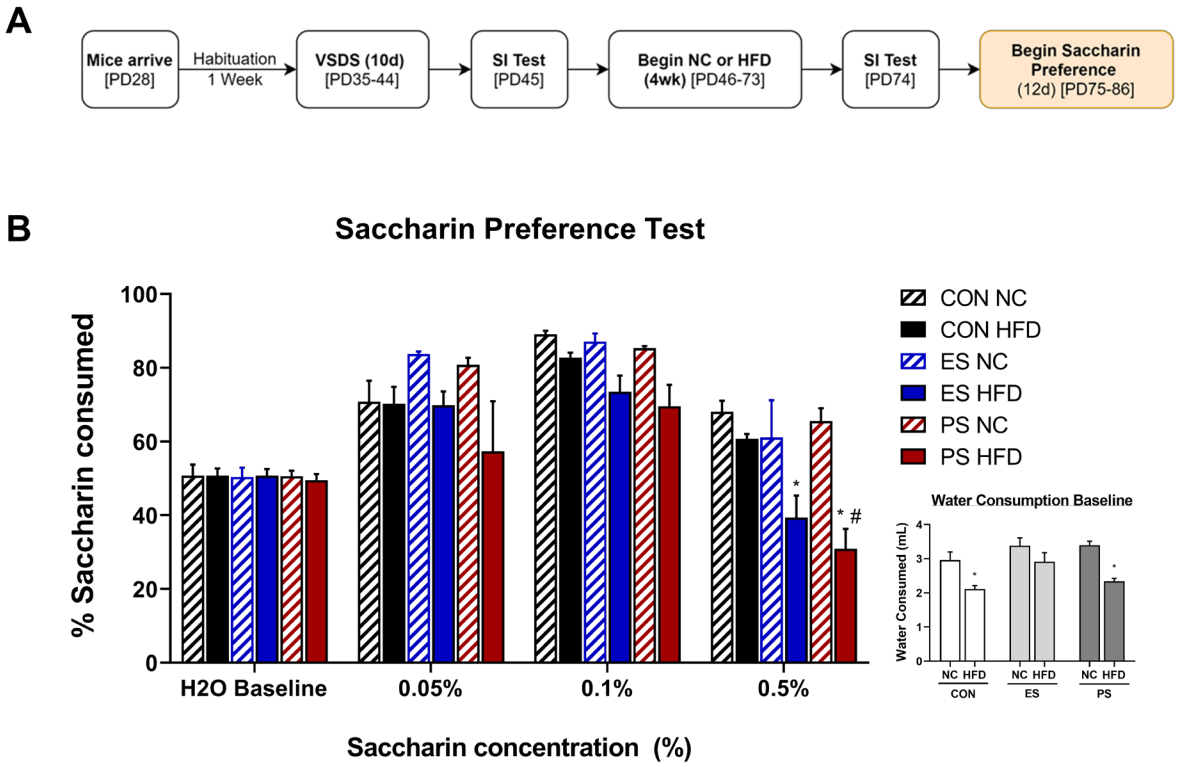
Figure 10: Long term social interaction after VSDS and HFD exposure.



A) Mice were retested in the social interaction test (SIT) 24 hr after 4 weeks of normal chow (NC)- or high-fat diet (HFD)- exposure (n=6/group). **B)** To evaluate the effect of 4 weeks of HFD exposure on social interaction, the mice were exposed to the social interaction test (SIT) 24 hours after the last day of diet exposure. Mice in the PS condition retained their defeated phenotype (i.e., social avoidance), as the PS+NC- ($p<0.01$) and the PS+HFD-exposed ($p<0.01$) were significantly different from the mice in the CON+NC and CON+HFD conditions with a main effect of stress ($p<0.05$). Although the mice in the ES condition showed moderate increases in social interaction from baseline after 1 month of diet exposure, those in the ES+HFD condition still showed significant reduction in social interaction ($p<0.05$) when compared to both the CON+NC- and the CON+HFD-exposed mice. $^{*}p<0.05$: significantly different than the CON-NC; $^{\#}p<0.05$:

significantly different than CON-HFD. All data are expressed as the mean ± SEM. Reprinted with permission from Sial et al., 2021.

Figure 11: Effect of VSDS exposure followed by HFD on preference on saccharin.

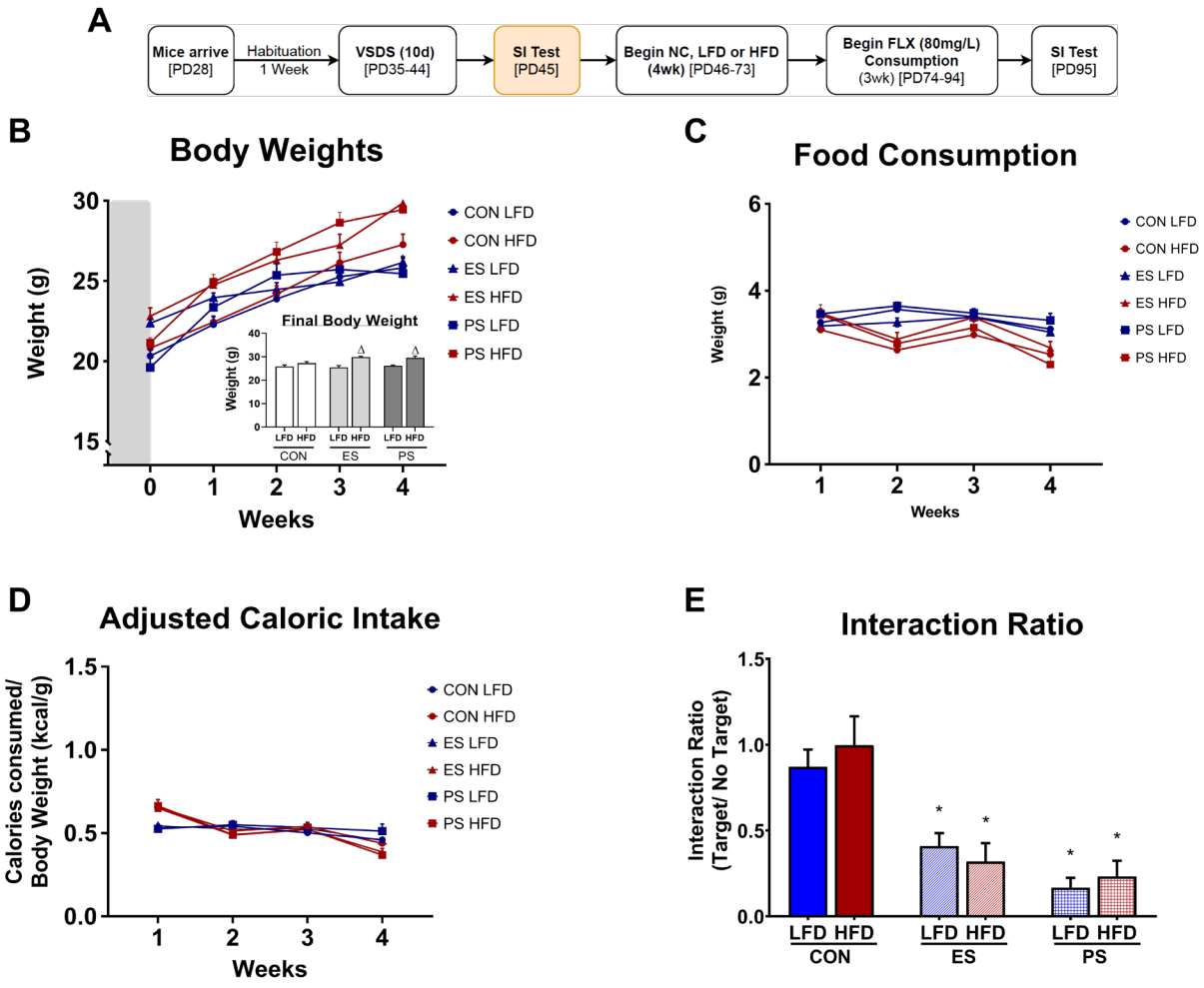


A) A separate group of adolescent mice were used to further assess the effects of VSDS+HFD exposure on reward sensitivity using a two-bottle saccharin preference test (n=6/group). **B)** There were no differences between any group in percentage of water consumed at baseline. In addition, no significant differences were observed at the 0.05% and 0.1% saccharin concentration. However, at the 0.5% concentration, both the ES+HFD- and PS+HFD-exposed mice consumed significantly less saccharin as compared to the CON+NC group (post hoc, $p<0.05$). A MANOVA revealed differences in preference for the sweetened solution as a factor of saccharin concentration (*main effect;* $p<0.05$; and diet (*main effect;* $p<0.05$). Only the PS+HFD-exposed mice showed a significant difference when compared to the mice in the CON+HFD condition (post-hoc $p<0.05$). Inset graph shows the raw consumption during the water baseline. A two-way ANOVA revealed

that the CON+HFD- and the PS+HFD-exposed mice drank less water than their NC-counterparts (post-hoc $p<0.05$). Mice in the ES+HFD condition trended towards a decrease, but this was not significantly different from the ES+NC-exposed mice (post-hoc $p>0.05$). Despite the HFD-induced adipsia, the relative percentage of saccharin consumed remained lower in the stressed group exposed to HFD. These findings indicate that concurrent stress and HFD exposure results in dysregulated sensitivity to reward (i.e.., saccharin), independent of the caloric value of the sweetened solution.

$^{*}p<0.05$: significantly different from CON+NC; $^{\#}p<0.05$: significantly different from CON+HFD. Reprinted with permission from Sial et al., 2021.

Figure 12: Effects of VSDS exposure prior to 4 weeks of LFD or HFD on social interaction, body weight, food intake, and caloric intake.

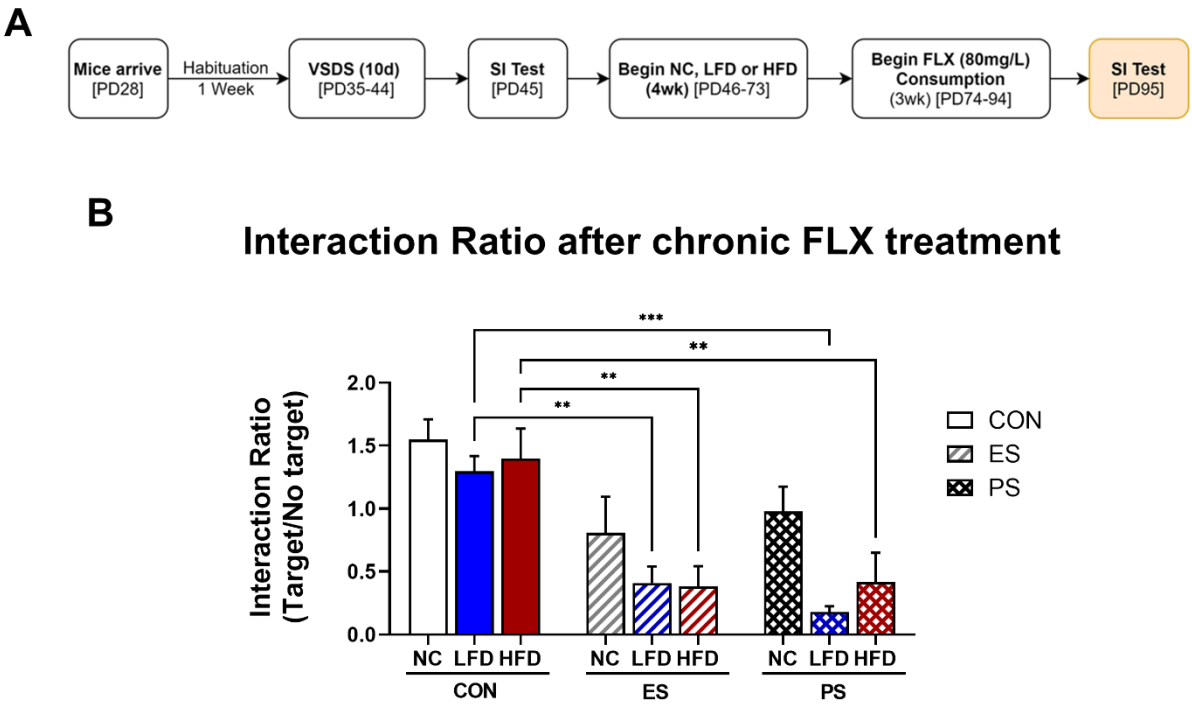


(A) Adolescent mice underwent VSDS and subsequently tested for social interaction. **(B)** Mice in the ES+ and PS+HFD conditions gained weight rapidly and were significantly heavier than the mice in the CON+LFD condition during the final week of diet exposure (post-hoc; $p<0.05$) with an interaction effect between stress, diet, and time ($p<0.05$). There was no difference in body weight between the CON+LFD- and the CON+HFD-exposed mice. **(C)** There were no differences in adjusted caloric intake between the groups in the final week of diet exposure (post-hoc $p>0.05$).

Novelty-induced hyperphagia in the first week results in a main effect of time ($p<0.05$) and diet ($p<0.05$) **(D)** Mice in the ES+LFD ($p<0.001$) and the ES+HFD conditions (post-hoc $p<0.05$) showed an interaction ratio significantly lower than their CON-counterparts. The PS+LFD- and the PS+HFD-exposed mice also showed a significant reduction in social interaction compared to their respective CON-counterpart mice (post-hoc $p<0.05$, respectively). There was no difference between the interaction ratio of LFD and HFD exposed mice in either ES or PS groups. All data are expressed as the mean ± SEM.

$^{*}p<0.05$: significantly different than their respective LFD-exposed counterparts. Reprinted with permission from Sial et al., 2021.

Figure 13: VSDS exposure before WSD during adolescence attenuates response to fluoxetine.



(A) To assess the antidepressant efficacy of fluoxetine (FLX) after VSDS and WSD exposure, mice received FLX (80mg/L) in the drinking water for 21 days. **(B)** Mice in the ES condition that received either LFD or HFD showed an attenuated response to FLX, as the interaction ratio was significantly lower from the mice in the CON+NC condition. Similar effects of attenuated FLX response were seen for the mice in the PS condition regardless of diet exposure (main effect; diet $p<0.01$ and stress $p<0.0001$).

Data are shown as mean ± SEM. $^{*}p<0.05$ $^{**}p<0.01$. Reprinted with permission from Sial et al., 2021.

CHAPTER IV
ALTERATIONS IN METABOLIC REGULATION AND MICROBIOME COMPOSITION ASSOCIATED WITH VICARIOUS SOCIAL DEFEAT STRESS AND HIGH FAT DIET DURING ADOLESENCE

Introduction

Despite their being compelling evidence highlighting the reciprocal relationship between diet-induced obesity and depression during adolescence (Dockray et al., 2009; Hammerton et al., 2014; Luppino et al., 2010; Marmorstein et al., 2014; Sanchez-Villegas et al., 2013; Singh et al., 2019), there is a sparsity of studies investigating the underlying mechanisms behind this comorbidity, commonly referred to as the metabolic-mood syndrome. One of the major purported contributors to the metabolic-mood syndrome is the consumption of globally popular western-style diets (WSD), which are hypercaloric with elevated concentrations of saturated fats and high glycemic index carbohydrates (Drewnowski and Popkin, 1997; Shakersain et al., 2016). The other major element is chronic stress, which, if experienced during the sensitive period of adolescence, profoundly interferes with normal metabolic functioning, which can contribute to the development of psychiatric disorders and physiological complications later in life (Alastalo et al., 2013a, 2013b; Kessler et al., 2010). Preclinical researchers have started utilizing concurrent chronic stress paradigms and WSD exposure in an attempt to model the symptoms of the metabolic-mood syndrome (Chuang et al., 2011; Goto et al., 2016). This type of experimentation allows for the delineation of the various biological systems that have been implicated, including the neural reward system (Sharma and Fulton, 2013; Vendruscolo et al., 2010), inflammatory systems (Cheng et al., 2019), as well as hormonal and metabolic systems (Chuang et al., 2010a; Han et al., 2015; Sivanathan et al., 2015). Novel avenues of research have found that these broad metabolic and

psychiatric alterations may all be mediated either directly or indirectly through the gut-brain-axis, specifically the gut microbiota (Foster and McVey Neufeld, 2013; Rea et al., 2016; Sandhu et al., 2017).

Our gastrointestinal systems encompass the largest and most diverse aggregation of microbiota in the human body, with gut bacteria outnumbering human nucleated cells at a ratio of 10:1 (Pevsner-Fischer et al., 2016; Sender et al., 2016). These gut microorganisms are of critical importance, as they carry out a wide range of metabolic and immunologic functions, ranging from the production of short-chain fatty acids and dietary fibers to the synthesis of amino acids and promotion of various anti-inflammatory cytokines (Quigley, 2013). Cultivation of the human microbiome begins immediately after birth, growing and developing with the host and adapting to the internal environment (Sekirov et al., 2010). Adult microbiotas are fairly stable in that approximately 60% of strains are consistently present, with the remaining proportion fluctuating due to host- and environment-specific factors (Faith et al., 2013; Mehta et al., 2018). Factors like diet (Claesson et al., 2012), stress (Foster et al., 2017), and antibiotic usage (Marques et al., 2010), especially during childhood, can cause drastic alterations of the microbiome. While the capability of the microbiome to evolve in its response to various stimuli renders it versatile, large population shifts can result in gut dysbiosis, leading to pathological ailments including obesity and diabetes (Carding et al., 2015). For instance, the two largest groups of bacteria within our gut are Firmicutes and Bacteroidetes, which represent more than 90% of the total bacterial population (Qin et al., 2010). Changes in the ratio in Firmicutes to Bacteroidetes abundance have been associated with obesity and obesogenic phenotypes in both humans and rodents (Ley et al., 2006a; Mariat et al., 2009). Firmicutes is thought to increase caloric efficiency or the effectiveness of obtaining

nutrients from food, thus leading to greater weight gain (Krajmalnik-Brown et al., 2012). Bacteria such as Lactobacillus, Allobaculum, and Sutterella are known to be reduced by stress, whereas oral administration of probiotics such as Lactobacillus reuteri restored depressive-like behaviors (Xie et al., 2020). Mice that have gone through physical defeat stress also show a reduced abundance of the Clostridia species (Bharwani et al., 2016) whereas treatment with Lactobacillus rhamnousus is also known to block anxiety-like effects of social stress (Bharwani et al., 2017). Thus, these microbiota are a key regulator of the gut-brain axis, regulating neuronal, endocrine, and immune signaling (Salvo-Romero et al., 2020), providing a basis for the investigation into their contributions to other pathologic states, including substance abuse (Hofford et al., 2021) and response to stress (Rea et al., 2016).

The gut microbiome interacts with the central nervous system in several ways, the most predominant mechanisms being neuroimmune in nature. Dysregulated microbiomes can increase concentrations of proinflammatory cytokines (El Aidy et al., 2015), intake in fatty acids (Stallone, 1994), and serum corticosterone (El Aidy et al., 2015), which have been implicated in both obesity and depression (Kelly et al., 2016). Moreover, these immunologic effects extend to the brain, wherein inflammation induces breakdown of the blood-brain-barrier (BBB) (Braniste et al., 2014) and the consequent infiltration of macrophages, leading to neuroinflammation and the alteration of behaviors (Chan et al., 2019). Gut flora can also modify behavior via neurotransmitters and neuropeptides, as it has been shown to alter levels of GABA, L-glutamate, L-tyrosine, and L-tryptophan (Jenkins et al., 2016). Taken together, our current understanding of the gut microbiome reinforces the case for its involvement in the precipitation of depression, as monoamines and

glutamate are targets for classical and novel antidepressant treatments (Berton and Nestler, 2006; Sanacora et al., 2012).

Asynchronous utilization of diet-induced obesity and chronic stress models of depression has limited our ability to observe deficits induced by the interaction of the two insults when experienced concomitantly. The current evidence has not clearly outlined the effect of early life emotional/psychological or physical stress combined with WSD exposure on microbiome composition and the subsequent alterations in metabolism of neural substrates. To this end, the experiments I outlined here aimed to fill this gap by exposing adolescent mice to vicarious social defeat stress (VSDS) followed by exposure to either control diet (CD) or a high-fat and high carbohydrate diet (HFD) for four weeks. Microbiota composition was determined though 16S rRNA sequencing, and ELISA's were used for the stress hormone corticosterone and proinflammatory cytokine, Interleukin-6. I hypothesized that both emotional and physical stress will cause metabolic and microbiological alterations similar to HFD but that the combination of both will lead to synergistic deficits.

Methods

Animals

A total of 48 male C57BL/6J mice (C57; Jackson Labs, Bar Harbor, Maine), postnatal day (PD) 28 at time of arrival, and CD-1 retired breeders (Charles Rivers, North Carolina) were housed at 23°C, in clear polypropylene boxes, on a twelve-hour light/dark cycle (lights on at 7AM, lights off at 7PM) and habituated for 7 days. The C57 mice were group-housed during habituation and moved to single-housing (PD35) for the remainder of the experiment, while the CD-1 mice were singly housed from arrival. All experimental procedures were conducted in strict compliance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003).

Stress

Vicarious social defeat stress (VSDS) was performed as described previously. Adolescent mice were randomly assigned into control (CON), emotional (ES), or physical stress (PS) conditions and exposed to 10 days of VSDS lasting ~10 minutes each. Briefly, the home cage of the CD-1 mouse was separated into two compartments by a perforated clear Plexiglas divider. During the daily stress sessions, the intruder mouse (PS) was placed into the territorialized home-compartment of a CD-1 and subsequently physically defeated. At the same time, a different experimental mouse (ES) in the adjacent compartment witnessed the defeat bout, mimicking a form of emotional social stress. At the end of the defeat session, the ES-exposed mouse moved to stay overnight in an adjacent cage that was not used that day for defeats. The PS-exposed mouse was placed overnight in the compartment adjacent to the CD-1 that socially defeated it, for

overnight sensory exposure. To minimize physical injury, the daily sessions were terminated when the intruder mouse adopted a persistent submissive posture, or when the CD-1 displayed excessive physical aggression. Mice in the CON condition were moved to new compartments daily without contact with a CD-1.

Diet

After the VSDS exposure, mice were further randomized to receive either control diet (CD; Research Diets, D12450K; fat content 10% kcal from synthetic oils, carbohydrate content 70% kcal from starch; 3.82 kcal/g) or a diet high in both fats and carbohydrates (HFD; Research Diets; D12451; fat content 45% kcal from lard, carbohydrate content 35% kcal from sucrose; 4.73 kcal/g) ad libitum (see Table 2). Micronutrient content was controlled between the diets. All food was placed in metal food hoppers as previously described.

Social Interaction Test

The social interaction test (SIT), a behavioral paradigm used to assess social avoidance, is used as the primary behavioral outcome measure after exposure to the VSDS paradigm. The SIT was performed 24 hours after the last defeat session. Briefly, the SIT is composed of two, 2.5 minute-sessions. In the first session, a mouse is allowed to explore an open field arena (40 cm × 40 cm) containing an empty wire mesh cage (i.e., no social target present). For the second session, the mouse is then removed, and a novel CD-1 mouse is placed into the wire mesh cage (i.e., social target present). The test mouse is returned to the arena and the amount of time spent in the "interaction zone" (8 cm wide corridor surrounding the wire mesh cage), as well as the time spent in the corners farthest from the social target is measured. The VSDS-defeated mice explore the

interaction zone significantly less when a target mouse is present and spend more time in the corners. Social interaction is calculated as a ratio of time spent in the interaction zone, with and without the target present (time in interaction zone with target/time in interaction zone without target). Interaction ratio below 1.0 indicate social avoidance and susceptibility to stress.

Microbiome Sequencing

Fresh fecal samples were collected directly into a cooled 1.5mL centrifuge tube, flash frozen on dry ice, and stored at −80 °C. DNA from fecal samples were isolated using DNeasy PowerSoil Kit (Qiagen) following kit instructions with a bead beating step. DNA concentration was determined with a NanoDrop1000. 48 samples (with the best DNA concentration and quality) in total, (6 groups as follows: CON-CD (n=9), CON-HFD (n=9), ES-CD (n=7), ES-HFD (n=7), PS-CD (n=8) and PS-HFD (n=8)) were sent to LC Science (Houston, TX) and amplification of the V3 and V4 region of the 16S rRNA bacterial genome was sent for sequencing (NovaSeq platform paired-end reads [2 x 250 bp]) (Callahan et al., 2016). After sequencing, amplicons are merged, chimera filtered, and quality controlled and denoised with dereplication to increase data accuracy and resolutions of species. Operational Taxonomic Units (OTUs) are subsequently constructed to allow for analysis of diversity assessment species annotation, classification, and differential analysis. OTU counts are also used to create the Shannon index of alpha diversity and principle coordinates analysis (PCoA) plots were generated using the Unifrac distance as an assessment of beta diversity using QIIME2 software (Bolyen et al., 2019).

ELISAs

Corticosterone Assay

The same group of mice were sacrificed, and trunk blood was collected into a 1.5mL centrifuge tube and allowed to sit at room temperature for 20 minutes. The whole blood samples were centrifuged for 1500g for 30 min at 4°C. The serum supernatant was pipetted into a clean tube for use in the corticosterone enzyme immunoassay kit as per manufacturer's instructions (Arbor Assays; K014-H1). Serum was diluted 1:100 using the provided buffer, pipetted into a 96-well plate, and allowed to incubate for 1 hour. The plate was washed with buffer, developed, and the colorimetric changes were measured using a plate reader (BioTek).-Serum corticosterone was calculated by comparing these values to optical density values obtained from corticosterone standards.

Interleukin-6 Assay

The same group of mice were sacrificed, trunk blood was collected into a 1.5mL centrifuge tube and allowed to sit at room temperature for 20 minutes. The whole blood samples were centrifuged for 1500g for 30 minutes at 4°C. The serum supernatant was pipetted into a clean tube for use in the interleukin-6 (IL-6) enzyme immunoassay kit as per manufacturer's instructions (BD; 550950). Serum was diluted 1:2 using the provided buffer and pipetted into a 96-well plate and allowed to incubate for 2 hours. The plate was washed with buffer, developed, and the colorimetric changes were measured using a plate reader (BioTek).-Serum IL-6 was calculated by comparing these values to optical density values obtained from IL-6 standards.

Statistical analyses

Data were analyzed using GraphPad Prism (version 9) software. When appropriate, two-way ANOVAs or Student's t-tests were used to determine statistical significance of pre-planned comparisons. Data are expressed as the mean ± SEM, with statistical significance set at $p<0.05$.

Results

Social interaction after early-life VSDS exposure.

Adolescent mice (PD35) were exposed to VSDS for 10 days and subsequently tested in the social interaction test (SIT) to measure avoidance (PD45; Fig. 14*A*) One-way ANOVA revealed that mice exposed to either emotional (ES; n=14) or physical stress (PS; n=16) exhibited a decrease in interaction ratio ($F_{(2,45)}$=11.39, p<0.0001; see Fig. 14*B*) when compared to non-stressed control mice (CON; n=18).

Early-life stress and high fat diet alters microbiota composition.

After VSDS exposure, mice were randomly assigned into control diet (CD) or high-fat diet (HFD), yielding 6 groups: CON-CD (n=9), CON-HFD (n=9), ES-CD (n=7), ES-HFD (n=7), PS-CD (n=8) and PS-HFD (n=8). Beta diversity as seen in the PCoA plot (Fig. 15*A*) indicates population overlap between the groups. Alpha diversity, or the diversity within the local ecosystem, demonstrated significant differences as a factor of diet ($F_{(1,42)}$= 14.57, p<0.001; Kruskal-Wallis p=0.0078) as seen in the Shannon Index violin plot (Fig. 15*B*). Both CON-HFD and PS-HFD showed increased diversity compared to their CD-fed counterparts (pink and mustard vs orange). Composition and expression of species within groups can be observed in the phylum relative abundance stacked chart (Fig. 15*C*). Specifically, Actinobacteria ($F_{(1,42)}$=9.575, p<0.01) and Firmicutes ($F_{(1,42)}$=25.49, p<0.0001) have alterations as a factor of HFD exposure in adolescent mice.

Observed changes in Firmicutes to Bacteroidetes ratio in response to stress and diet.

Firmicutes to Bacteroidetes ratio was calculated from relative abundance at the phyla level (Fig. 16). Two-way ANOVA revealed a significant effect of stress ($F_{(2,46)}$=3.481; p<0.05) and diet

($F_{(1,46)}$=6.521; p=0.01) on the F:B ratio. Post-hoc analysis revealed that the PS-HFD-exposed mice have a significantly larger F:B ratio compared to CON-CD mice (p<0.05).

Heat map of major predicted altered metabolic pathways after VSDS and HFD.

The effect of stress and diet on the flora's relative metabolic activity was calculated using a group average of raw operational units (OTU) values from Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) and depicted in heatmap showing the SEM of each group. 56 pathways that showed the most alteration across groups were represented in the heatmap (Fig. 17).

Representative amino acids pathways altered by stress and diet in flora.

Select amino acid biosynthesis pathways were chosen to evaluate alterations in response to VSDS and CD or HFD. The OTU data from PICRUSt2 analysis was used to perform the analysis. Two-way ANOVA revealed that L-tryptophan biosynthesis was significantly dysregulated as a factor of diet ($F_{(1,42)}$=17.32; p<0.001; Fig. 18*A*). Post-hoc analysis demonstrated that the CON-HFD- (p<0.01) and PS-HFD- (p<0.05) mice had significantly higher levels of L-tryptophan biosynthesis than CON-CD-exposed mice. L-glutamate/glutamine was significantly different between diets ($F_{(1,42)}$ = 8.442; p<0.001; Fig. 18*B*). Post-hoc analysis showed that the CON-HFD- had had increased levels of L-glutamate/glutamine compared to the CON-CD-exposed group. Similarly, L-glutamate degradation was also affected by diet ($F_{(1,42)}$ = 17.43, p<0.001; Fig. 18*C*), however, post-hoc did not reveal any differences between groups.

Evaluation of serum corticosterone and interleukin-6 levels after VSSDS and HFD.

Trunk blood was collected, and serum was extracted for use in immunoassays (ELISAs). Serum corticosterone was measured in mice exposed to VSDS and either CD or HFD (n=7/group). A two-way ANOVA demonstrated that corticosterone (CORT) was influenced by stress ($F_{(2,34)}$ = 8.981; p<0.001; Fig. 19*A*), with post-hoc analysis indicating a significant increase in CORT in the PS-CD- compared to the CON-CD-exposed mice (p<0.05). Stress also significantly influenced Interleukin-6 concentrations ($F_{(2,33)}$= 4.264; p<0.05; Fig. 19*B*). Post-hoc analysis did not show statistically significant differences between the groups, but the PS-HFD-exposed group did trend towards an increase (p=0.0559).

Discussion

The relationship between obesity and mood disorders has been demonstrated. Two major contributors to this metabolic-mood dysregulation are the consumption of western-style diets (WSD), high in calories and elevated concentrations of saturated fats and high glycemic index carbohydrate, and stress (Drewnowski and Popkin, 1997; Shakersain et al., 2016). Stress, especially when experienced chronically during adolescence, profoundly interferes with normal metabolic functioning, contributing to the development of psychiatric disorders and physiological complications throughout life (Alastalo et al., 2013a, 2013b; Kessler et al., 2010). These metabolic and psychiatric alterations can be mediated through the gut-brain-axis, specifically the gut microbiota (Foster and McVey Neufeld, 2013; Rea et al., 2016; Sandhu et al., 2017). Gut microorganisms carry out metabolic and immunologic functions, ranging from production of fatty acids and dietary fibers to the synthesis of amino acids and promotion of anti-inflammatory cytokines (Quigley, 2013). The current evidence, however, has not clearly outlined the effect(s) of early life emotional or physical stress combined with WSD exposure on microbiome composition and the subsequent alterations in metabolism of neural substrates. The findings presented here, to my knowledge, demonstrate for the first time that exposure to early-life stress and a high-fat and high carbohydrate diet (HFD) causes major alterations in microbiome composition, predicted microbiome function, and immunologic blood markers. The relationship between depression and obesity (Skilton et al., 2007) is well-established; however, the ability to selectively distinguish between the overlapping symptomatology will likely create new avenues in the development of more targeted treatments. Here, I exposed adolescent mice to vicarious social defeat stress (VSDS) followed by exposure to either control diet (CD) or high-fat/high-carbohydrate (HFD) for four weeks. Along with the overall aim of these experiments, the use of a micronutrient-controlled diet

(CD) ensured that the differences observed are a factor of either the high fat/high carbohydrate content or the stress, whether emotional/psychological (ES) or physical (PS).

Microbiota composition was determined via 16S rRNA sequencing and uncovered substantial overlap between the groups, indicating that a majority of bacterial taxa present were shared between all groups. Alpha diversity, or diversity within the local ecosystem, was significantly altered between diet and stress conditions. Exposure to HFD increased the alpha diversity, whereas stress did not appear to have an effect; indicating that the diet allowed for a greater variety of bacteria present, though further analysis is needed to determine if these bacteria are pathogenic in nature. These date are corroborated by recent studies which have shown that chronic social defeat stress (CSDS) does not induce changes in alpha diversity in adult mice (Xie et al., 2020), whereas HFDs increase alpha diversity; however, this phenotype has not been consistently observed and may be dependent on the strain of mouse and specific composition of the diet (Bisanz et al., 2019).

Previous studies found that shifts in taxa composition at the phyla level are associated with gut dysbiosis; thus, I elected to compare the relative abundance at this level. Across groups, Firmicutes, Actinobacteria, and Bacteroidetes were the most abundant taxa found, with Verrucomicrobia, Proteobacteria, and Patescibacteria accounting for most of the lowly-expressed bacteria. Drastic shifts were seen in the major taxa in that HFD-exposure led to decreased Actinobacteria abundance and increased Firmicutes abundance. To evaluate the gut health, the Firmicutes and Bacteroidetes OTUs were used to calculate a ratio (F:B ratio). The F:B ratio acts as a measure for normal gut status in humans, where a higher ratio is associated with obesity and a lower ratio is associated with weight loss (Ley et al., 2006b; Mariat et al., 2009). Here, I observed an increase in the F:B ratio as a factor of both stress and diet, an effect that was pronounced in the

PS+HFD-exposed mice, finding highlighting the synergistic deficits caused by both physical stress and diet. Interestingly, however, in this paradigm, exposure to emotional stress was not sufficient to induce these effects. The presence of anaerobic bacteria within the gut is crucial for proper functioning, as they allow for both the translocation of normal intestinal bacterial but also the prevention of pathogenic bacteria colonization (Wells et al., 1988). Both the ES+HFD and PS+HFD-exposed groups exhibited decreases in anaerobic bacteria, indicating that HFD worsened consequences for both psychological and physical stress, whereas the stressors alone had no effect.

To evaluate how the metabolic functioning of the biome may be disrupted, PICRUSt2 analysis was performed to demonstrate predicted functions based on sequenced marker genes (Douglas et al., 2020). Thousands of homologous genes and enzymes were categorized into ~340 families and the top 56 most dysregulated pathways were plotted as a heatmap. The observed differences in bacterial abundance between diet+stress groups were primarily in amino acid metabolism, nucleotide biosynthesis, and sugar metabolism. Most of the differences (~90%) were seen between the mice in the CD and HFD conditions, independent of stress. One possibility for this result may be that consumption of HFD robustly influences the structure and composition of the gut microbiota, so much so that it may be masking any effects of the stressors. Only the super-pathway of the adenosine nucleotide metabolism was dysregulated by PS and HFD exposure. Previous studies have shown that purine metabolism is severely dysregulated in mice that are susceptible to physical defeat stress (Hamilton et al., 2020). This suggests that HFD may be worsening the metabolic outcomes in PS-exposed mice, though it is unclear why the effect was not observed in PS+CD-exposed group. However, the bacteria in the PS-exposed mice did show deficits in pyruvate metabolism, which could influence intracellular signaling via cellular energetics (Engelking, 2015) and inositol degradation (MacFarlane and Di Fiore, 2018). Given the

involvement of amino acids like glutamate and tryptophan directly and indirectly in the manifestation of depressive disorders (Charney, 1998; Sanacora et al., 2012), I sought to investigate the metabolic pathways of these residues. Exposure to HFD increased L-tryptophan biosynthesis levels in CON-exposed mice, but this effect was blocked by stress exposure. This effect was similarly observed for L-glutamate/glutamine biosynthesis. L-glutamate degradation was increased in all groups receiving HFD. Interestingly, it is unknown whether dysregulated amino-acid metabolic activity of these microbes directly influences neurotransmitter levels within the brain (Jenkins et al., 2016). One hypothesis is that these changes influence signaling of the gut to the brain via the vagal nerve (Fülling et al., 2019). However, the gut has been shown to modulate peripheral tissues via a number of mechanisms, including the alteration of stress hormone levels (Salvo-Romero et al., 2020) and inflammatory markers (Kelly et al., 2016).

To determine whether stress hormones like corticosterone (CORT) are influenced by stress and diet exposure, I collected serum from mice who underwent early-life stress and consumed HFD for 1 month. I investigated CORT specifically because its proper regulation is known to be crucial for proper functioning of systems that modulate mood (Warren et al., 2013; Zhao et al., 2008), as well as the response to diet (Guarnieri et al., 2012; Karatsoreos et al., 2010). I hypothesized that circulating levels of CORT would be higher in the stress-exposed groups and that HFD exposure would amplify this increase. This hypothesis was partially confirmed, as CORT was significantly altered by stress; however, only the PS+CD-exposed group was significantly different than the CON+CD-exposed mice. Previous studies have shown that both ES and PS result in long-term increases in CORT (Warren et al., 2013), though the effect was not observed in the current ES-exposed group. I postulate that low-power and the time of day of serum collection may have influenced these results, as CORT levels varies depending on the time of day (Sage et al.,

2001). My hypothesis that the increase in CORT would be amplified with HFD was not supported, as these groups were not significantly different. Given that VSDS can lead to subsets of susceptible and resilient mice, I will likely need more power to isolate these subsets and run them independently, which would yield data with greater resolution (Sial et al., 2016). CORT dysregulation has also been associated with immune dysfunction (Anisman et al., 2008; De Bosscher and Berghe, 2000), which I partially characterized by monitoring interleukins.

To characterize downstream immunologic/inflammatory effects of the diet/stress manipulations, I performed ELISAs on proinflammatory cytokine interleukin-6 (IL-6). IL-6 has been shown to be dysregulated in depressive disorders (Maes et al., 1995) as well as stress-induced models of depression (Hodes et al., 2016). Additionally, IL-6 dysregulation has been implicated in obesity and other metabolic syndromes (Eder et al., 2009), whereas exercise-induced reduction of visceral fat is associated with decreased levels of IL-6 (Wedell-Neergaard et al., 2019). Given the previous evidence indicating that CSDS exposure increases IL-6 levels (Hodes et al., 2014), I hypothesized that IL-6 would increase in all stressed groups, with HFD compounding this elevation. This hypothesis was partially supported as stress did have a significant effect on IL-6; there appeared to be a trend as PS+HFD-exposed mice exhibited the greatest level of inflammation (based on IL-6). The long term effects of stress-induced increases in proinflammatory cytokines like IL-6 are unknown, but HFD has been shown to increase IL-6 levels (Cortez et al., 2013), complicating the picture. My data suggests that stress-induced inflammatory effects may be long-term and may be potentiated by HFD-exposure.

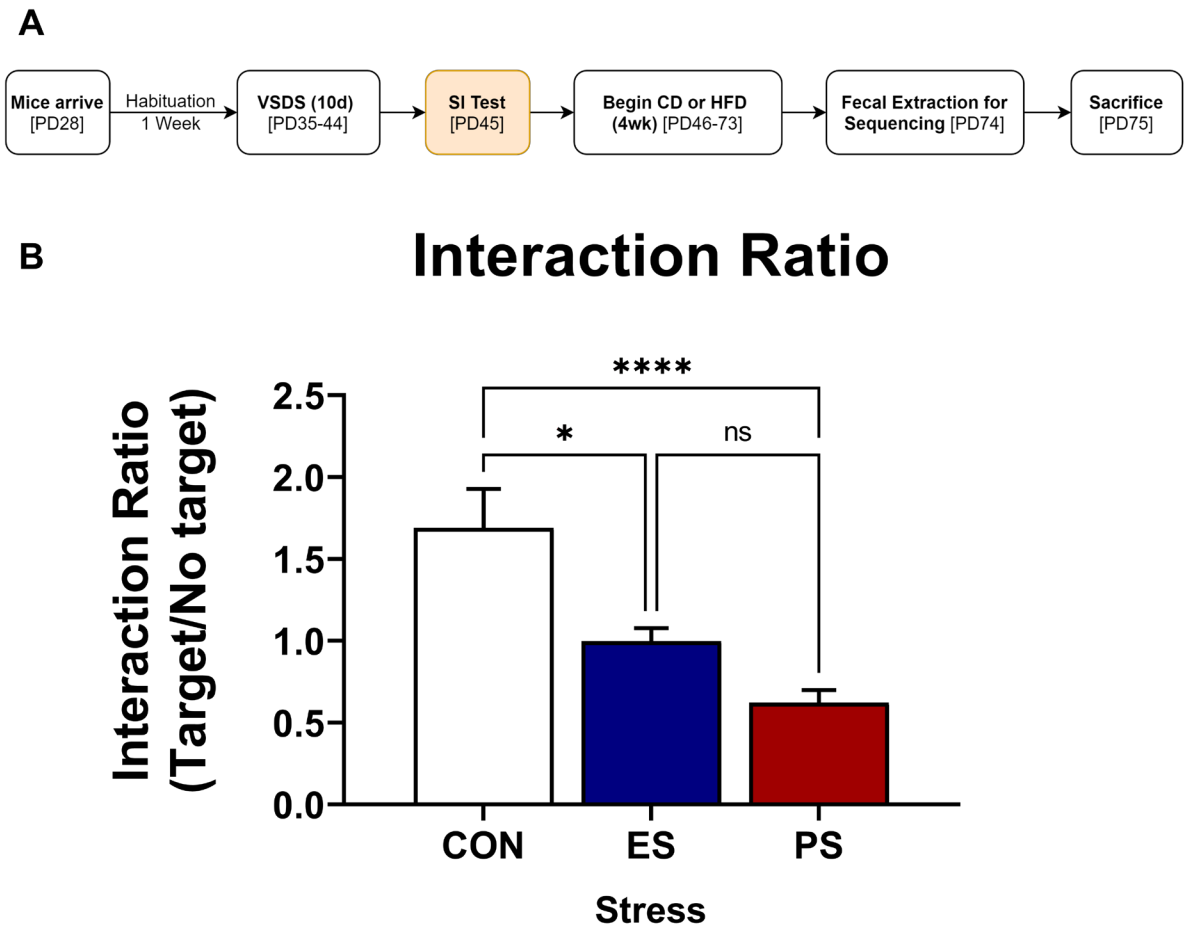
There are several limitations of this study. For instance, although there is evidence suggesting that females may be more susceptible to the metabolic-mood comorbidity (Lin et al., 2014), I was unable to use female mice due to the intrinsic limitations of the CSDS and VSDS

paradigms. Additionally, fecal samples may not be indicative of cecal bacterial composition. Studies comparing the microbiota composition of the proximal and distal large intestine would likely enhance our knowledge of the subject by providing more information on how the microbiome changes based no relative location within the gut itself. Furthermore, the study was low-powered, and the mice exposed to stress were not separated by resiliency or their susceptibility to stress. Being able to distinguish these subsets may reveal even greater differences between groups in terms of their metabolic, lipid, and protein profiles (Hamilton et al., 2020). Another limitation lies in the variation observed between the experimental groups, which could be mitigated by group-housing the mice, which would lend to confounding cage effects (McCafferty et al., 2013; Wang and LêCao, 2020).

In conclusion, I demonstrated that stress and diet have profound effects on microbiome composition, the stress hormone corticosterone, and the pro-inflammatory cytokine IL-6. Consumption of HFD resulted in gut dysbiosis as evidenced by differences in the Firmicutes: Bacteroidetes ratio. Diet also induced major changes in the metabolic pathways used by the bacteria to maintain normal homeostasis, as evidenced by serum corticosterone and IL-6 changes, which also exhibited stress-induced deficits. Further studies elaborating on the effects of HFD and stress concurrently may allow for a better understanding and enhancement of potential therapeutic options for the metabolic-mood syndrome, such as probiotic, which have previously been shown to relieve depression symptoms (Gawlik-Kotelnicka and Strzelecki, 2021). With these findings, I hope to have provided a new perspective in the understanding of disorders that have historically been treated as separate entities, ultimately inspiring new avenues of investigation for the development of novel and targeted nutraceuticals.

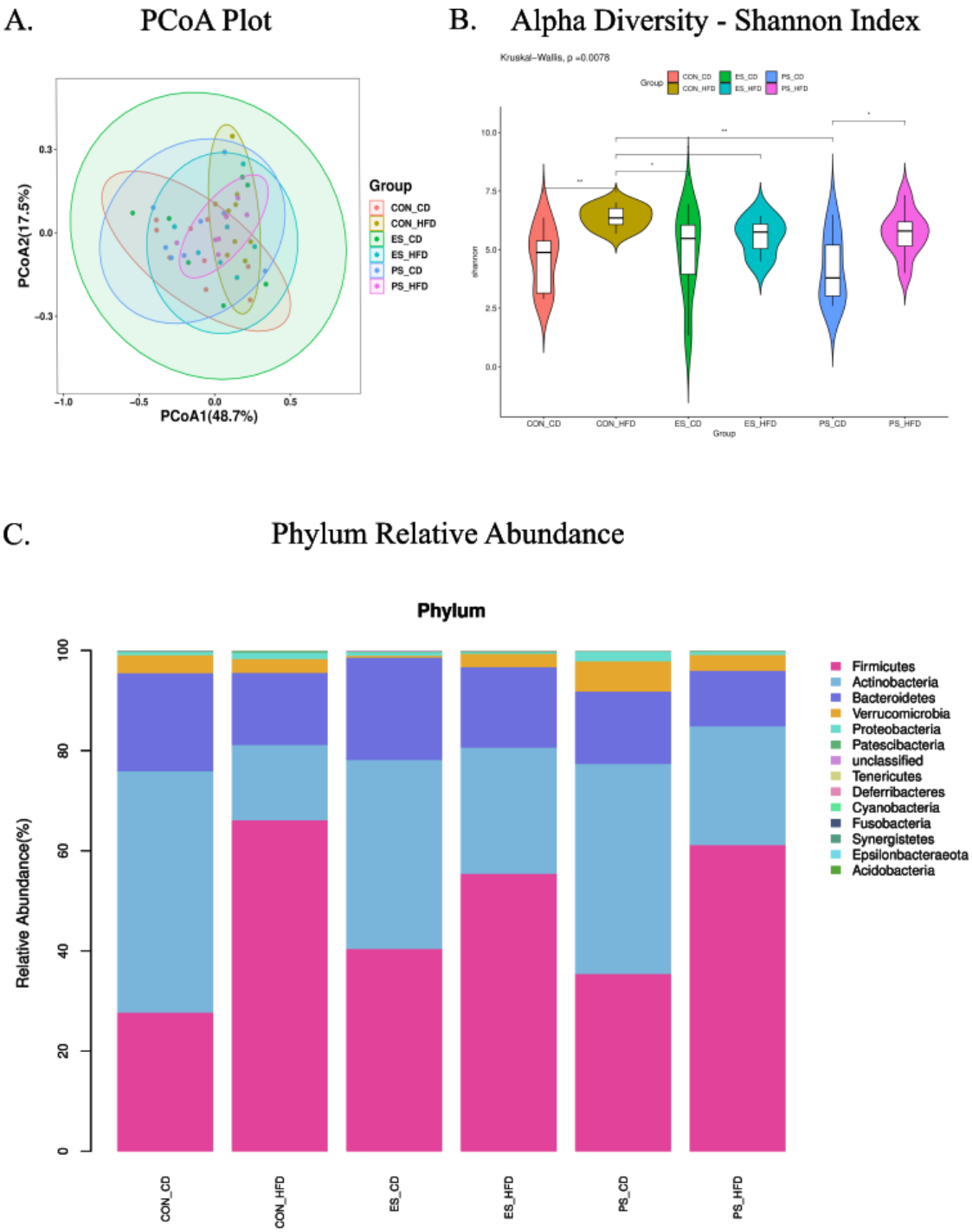
Figure

Figure 14. Social interaction after early-life VSDS exposure.



A) Adolescent (PD35) mice were exposed to no-stress (CON; n=18), emotional stress (ES; n=14), or physical stress (PS; n=16) for 10 days and subsequently tested in the social interaction test 24 hours after the last defeat (PD45). **B)** Mice exposed to either ES ($p<0.05$) or PS ($p<0.001$) exhibited a decrease in social interaction when compared to the non-stressed control mice (CON). Data are presented as a ratio of the total time spent with a social target to the total time spent in the interaction zone without a social target. ($^{*}p<0.05$, $^{****}p<0.001$)

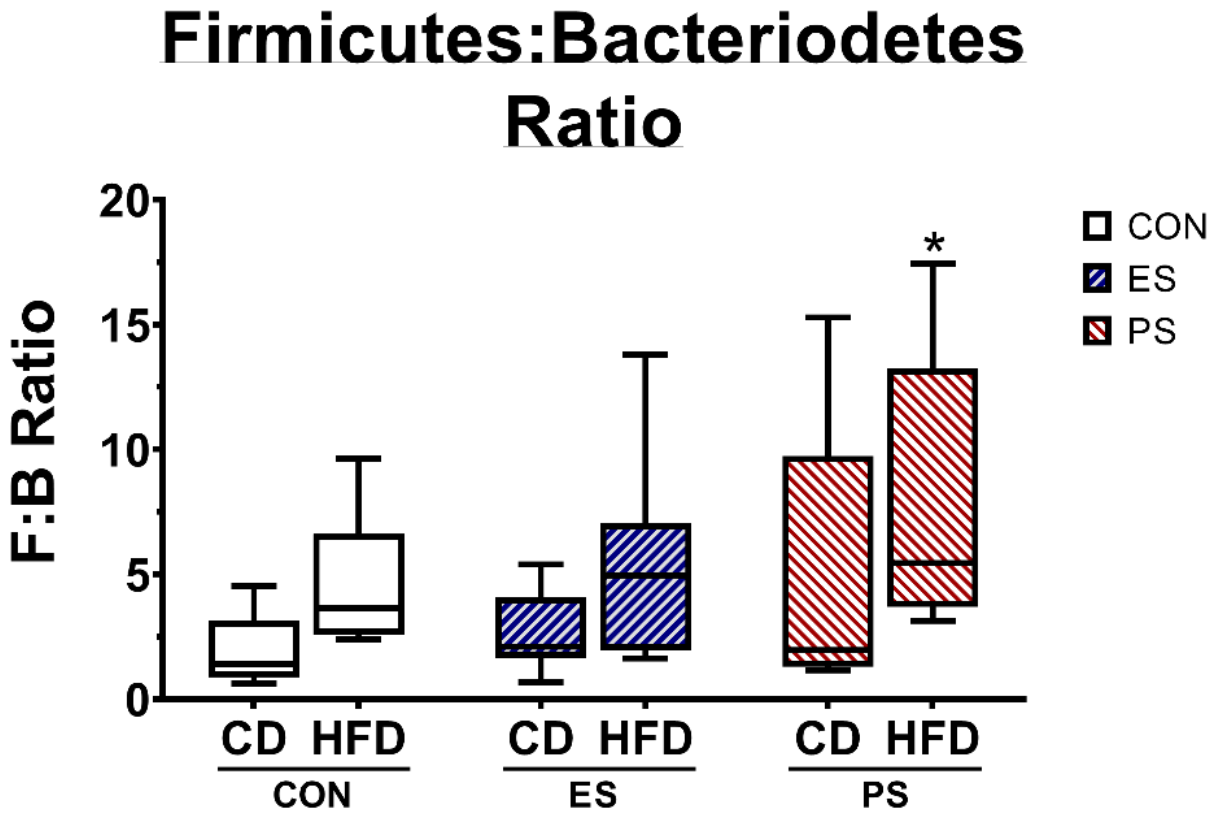
Figure 15. Early life stress and high fat diet cause alterations in microbiota composition.



A) Early-life stress, whether physical or emotional, and high-fat diet exposure cause a wide range of between-subject variation, as evidenced by population overlap in the weighted Unifrac distance

principal coordinates analysis (PCoA) plot. **B**) Alpha or local diversity is shown in the Shannon index. High-fat diet exposure in CON and PS groups increases alpha diversity ($p<0.05$). **C**) Relative abundance of the 14 highest taxa distribution is shown, with the larger proportion representing a higher abundance. Different colors represent different taxa and columns represent different groups. HFD induced an increase in Firmicutes and a decrease in Bacteroidetes.

Figure 16. Observed changes in Firmicutes to Bacteroidetes ratio in response to stress and diet.

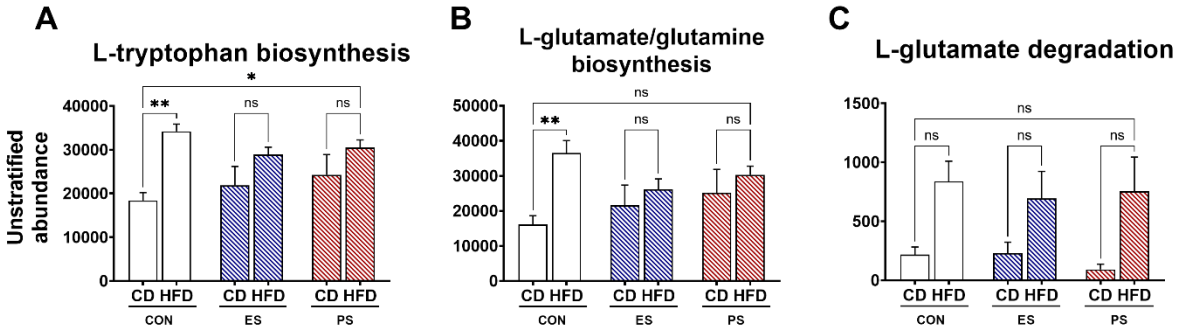


The ratio of Firmicutes to Bacteroidetes (F:B) was increased as factor of stress and diet ($p<0.05$). Exposure to PS and HFD during adolescence led to a significant increase in F:B ($p<0.05$). Data are presented as a ratio (mean ± min to max).

Figure 17. Heatmap of major predicted altered metabolic pathways after VSDS and HFD.

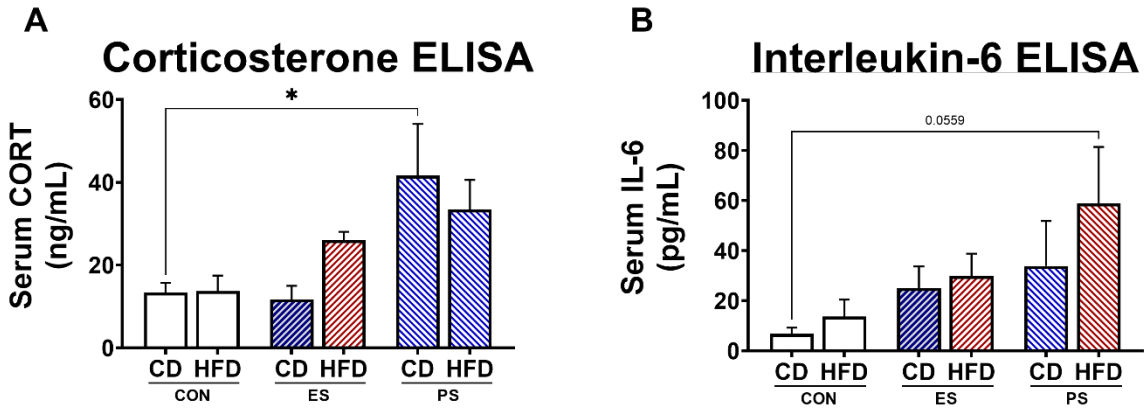
Heatmap displays the raw group average OTUs of predicted Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) in response to either no stress (CON), emotional stress (ES) or physical stress (PS) before control diet (CD) or high fat/high-carbohydrate diet (HFD). Various dysregulated pathways have been depicted on the rows and experimental groups are arranged in columns. The darker the color, the more likely the flora is associated with that related function. A majority of altered pathways are related to amino acid and nucleotide metabolism, as well as cellular respiration. Data are shown as SEM.

Figure 18. Representative amino acids pathways altered by stress and diet in flora.



From the heatmap PICRUSt2 data, select amino acid biosynthesis and degradation pathways were analyzed in response to either no stress (CON), emotional stress (ES) or physical stress (PS) before control diet (CD) or high fat/high-carbohydrate diet (HFD). L-tryptophan biosynthesis ($p<0.001$), L-glutamate/glutamine biosynthesis ($p<0.001$) and L-glutamate degradation ($p<0.001$) were significantly altered by diet, with post-hoc analysis revealing that the CON-HFD and PS-HFD groups both exhibited an increase in L-tryptophan biosynthesis in the flora compared to the CON-CD mice ($^{*}p<0.05$, $^{**}p<0.01$). Data are presented as unstratified abundance (mean ± SEM).

Figure 19. Evaluation of serum corticosterone and interleukin-6 levels after early-life stress and HFD- exposure.



We assessed changes in serum stress hormones (corticosterone; CORT) and cytokines (interleukin-6; IL-6) in response to no stress (CON), emotional stress (ES) or physical stress (PS) before control diet (CD) or high fat/high-carbohydrate diet (HFD). **A)** Significant changes in serum CORT were a factor of stress ($p<0.05$), with PS-CD mice demonstrating a significant increase in CORT compared to CON-CD mice ($p<0.05$). **B)** IL-6 levels in serum were also significantly altered by stress ($p<0.05$) and PS-HFD had a higher level of IL-6 compared to CON-CD ($p=0.05$). Data are presented as concentration in ng/mL or pg/mL respectively (mean ± SEM).

CHAPTER V

BIOCHEMICAL ASSESSMENT AND VIRAL MANIPULATION OF AKT IN THE CONTEXT OF CONCURRANT EARLY-LIFE STRESS AND WESTERN-STYLE DIET

Introduction

The incidence of adolescent obesity (Ogden et al., 2012) and depression (Mojtabai et al., 2016) has been rising over the past few decades. Given the comorbidity between these disorders (Mannan et al., 2016) and the heightened physiologic and psychologic vulnerability to stress during the sensitive period of adolescence, it is crucial to gain a better understanding of these conditions in order to develop early interventions that may prevent long-term complications. Most preclinical studies using rodent models have been unable to induce obesogenic phenotypes through diet within the short period of adolescence (~30 days) (Spear, 2000) and are therefore unable to fully mimic the clinical syndrome within the adolescent period. While studies have utilized models of diet-induced obesity (Speakman, 2019) and chronic stress paradigms during early-life to induce depressive-like phenotypes (Nestler and Hyman, 2010), very few have combined these approaches to delineate their shared symptomatology as the vast majority model these conditions in parallel. Recently, more studies have attempted to characterize the role of the brain in mediating the link between mood and metabolic disorders (Chan et al., 2019; Chuang et al., 2010b, 2011).

Limbic regions within the brain, including the nucleus accumbens (NAc) and the ventral tegmental area (VTA), are key regulators of reward and mood under normal conditions thus playing a role in the pathophysiology of psychiatric disorders (Nestler and Carlezon, 2006). More specifically, dysregulation of secondary messenger systems within these neural hubs contribute to abnormal behaviors (Niciu et al., 2013). Kinases, such as extracellular signal-regulated protein kinase-1/2 (*Erk1/2*), and other downstream targets of brain derived neurotrophic factor (BDNF)

(Numakawa et al., 2010) have been implicated in the development of the detrimental effects of stress (Gourley et al., 2008; Iñiguez et al., 2010b), and manipulation of *Erk* activity affects the efficacy of antidepressants such as fluoxetine (Iñiguez et al., 2014a). Interestingly, *Erk* activity has also been implicated in mediating leptin signaling within the hypothalamus, potentially contributing to obesity pathology (Balland et al., 2014). Other kinases, such as protein kinase B (*Akt*), are also involved in stress reactivity (Krishnan et al., 2008), with similar downstream targets to *Erk1/2* (e.g., CREB). Similar to *Erk*, *Akt* has been shown to mediate the effects of other antidepressants like the NMDA antagonist ketamine (Parise et al., 2021). *Akt* is also involved in the regulation of glycogen synthase kinase (*Gsk3β*), a fascinating target that is known to modulate stress-susceptibility, immune responses, and glucose homeostasis (Crofton et al., 2015a; S Han et al., 2016). Furthermore, *Akt* is part of the insulin-receptor substrate (IRS) pathway, which not only controls insulin signaling and the energy balance of a cell (Pardini et al., 2006), but also regulates cell size and function of dopamine (DA) neurons, thus influencing reward sensitivity (Russo et al., 2007). Despite our increasing understanding of these regulators, the role of these intracellular signaling markers within the limbic system have not been explored in the specific context of synergistic effects/deficits induced by early-life concomitant stress and poor diet exposure.

The VTA is the main dopaminergic hub of the mesolimbic system, projecting to the NAc to modulate reward-response and mood-related behaviors under normal conditions (Carlezon and Thomas, 2009; Galaj and Ranaldi, 2018). Though the intracellular mechanics that regulate the functional connectivity between these regions are important to characterize, delineating the cell-types in which these changes take place is paramount to understand the broader implications of these alterations. The NAc incorporates information from various brain regions to tune behavior through the distinct functions of DA type 1 (D1) and D2 receptors on GABAergic medium spiny

neurons (MSNs) (Calipari et al., 2016; Francis et al., 2015; Reynolds and Berridge, 2008; Richard and Berridge, 2011). For example, D1-containing MSNs are known to promote resilience to stressors, such as chronic social defeat stress (CSDS), whereas D2-MSNs appear to promote susceptibility to CSDS (Cao et al., 2010; Francis et al., 2015; Hodes et al., 2015). Molecular adaptations within these subset of cells alters stress response and overall behavioral output (Kronman et al., 2021), making it important to understand how regulators like *Akt* may be contributing to this overall behavioral effect. Viral-mediated gene transfer approaches have been used to manipulate gene expression within highly localized brain regions to tease apart specific functions (Iñiguez et al., 2010b). In fact, viral overexpression of *Akt* within the NAc induces a susceptible phenotype to stress in adolescent mice (Parise et al., 2021); therefore, I hypothesize that viral-mediated reduction of *Akt* would result in a resilient phenotype and that exposure to high-fat/high-carbohydrate diets (HFD) would block the effect of *Akt*. Furthermore, the NAc is heavily involved in the hedonic assessment and motivation for food and this can be affected by consumption of HFD (Kelley and Berridge, 2002; Naneix et al., 2017; Oginsky et al., 2016). This ability of NAc to mediate both stress- and diet-related phenotypical outputs makes it a highly attractive region to investigate in the metabolic-mood comorbidity.

Non-neuronal cell types, such as microglia, are far less explored despite their major roles of regulating the microenvironment of a brain region, modulating neuronal activity, and promoting cell survival (Badimon et al., 2020; Kalsbeek et al., 2016). As the resident macrophage of the brain, microglia are major contributors to neuroinflammation, which makes them ideal candidates for intervention for psychiatric disorders (Réus et al., 2015). Microglia-induced inflammation within regions like the hypothalamus is known to mediate obesity after HFD exposure, an effect blocked by microglial inactivation within the brain region. Inflammation within limbic regions such as the

NAc have been associated with depressive-like behaviors and alterations in food cravings (Décarie-Spain et al., 2018; Menard et al., 2017) that resemble abnormalities observed in metabolic-mood syndrome (Carter and Swardfager, 2016; Lutter and Elmquist, 2009). Given that microglia highly express *Akt* (Zhang et al., 2014), are responsive to stress stimuli (Stein et al., 2017), and demonstrate increased reactivity as seen through upregulated chemokine (c-c motif) ligand 2 (*Ccl2*); a target whose interactions with CCR2 allow for the recruitment of monocytes (Ataka et al., 2013; Cherry et al., 2020), I aimed to evaluate whether changes in intracellular signaling markers differed in microglia after simultaneous early-life CSDS and HFD exposure.

In the previous chapters, I have demonstrated that stress in combination with HFD exposure during adolescence leads to rapid weight gain and long-term negative behavioral consequences. Though many of the induced deficits were present during early adulthood (~PD70), it still did not allow for further testing during the adolescent period. To address this weakness, I exposed adolescent mice (PD35) to CSDS and HFD simultaneously for 10 days in order to expedite and truncate the paradigm to investigate the neurobiological effects of concomitant stress and diet exposure within the mesolimbic system. I first sought to characterize the involvement of *Akt* and *Erk* gene expression within the NAc and VTA after exposure to simultaneously CSDS+HFD. I hypothesized that HFD exposure would produce similar effects to CSDS exposure and that the combination of HFD and CSDS would cause synergistic deficits. I then sought to determine whether the changes in second messenger systems within the NAc were present in D1- or D2-containing MSNs using in-situ hybridization and whether non-neuronal populations such as microglia are involved in the manifestation of the phenotype.

Methods

Animals

Male C57BL/6J mice (Jackson Labs, Bar Harbor, Maine), postnatal day (PD) 28 at time of arrival and CD-1 retired breeders (Charles Rivers, North Carolina) were housed at 23°C in clear polypropylene boxes on a twelve-hour light/dark cycle (lights on at 7AM, lights off at 7PM) and habituated for 7 days. The C57BL/6J mice were group-housed during habituation and moved to single-housing at PD35 (the start of experimental manipulations), while the CD-1 mice were singly housed from arrival. This age was selected because it roughly coincides with early adolescence in humans (Abreu-Villaça et al., 2010; Andersen, 2003), a developmental stage of increased vulnerability to stress in which the adoption of poor eating habits and the onset of mood disorders often emerge (Banfield et al., 2016; O'Neil et al., 2014; Paus et al., 2008). Experimental procedures were conducted in strict compliance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council (US) Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003).

Stress

Chronic social defeat stress (CSDS) was performed as described previously (Berton and Nestler, 2006; Golden et al., 2011; Iñiguez et al., 2014a) with minor modifications. Adolescent mice were randomly assigned to a daily session of CSDS (10 days) lasting ~10 minutes each. Briefly, the home cage of the CD-1 mouse was separated into two compartments by a perforated clear Plexiglas divider. One side of the compartment temporarily housed the intruder mouse while the other housed the CD-1 retired breeder aggressor. During the daily sessions, the mouse intruder was placed into the territorialized home-compartment of a CD-1 mouse in the cage to the right of

the original housing and subsequently defeated. At the end of the defeat session, the mouse was placed overnight in the compartment adjacent to the CD-1 mouse that socially defeated it. To minimize physical injury, the daily sessions were terminated as soon as the intruder mouse adopted a submissive posture.

Diet

Mice were randomized to receive either control diet (CD; Research Diets, D12450K; fat content 10% kcal from synthetic oils, carbohydrate content 70% kcal from starch; 3.82 kcal/g) or a diet high in both fats and carbohydrates (HFD; Research Diets; D12451; fat content 45% kcal from lard, carbohydrate content 35% kcal from sucrose; 4.73 kcal/g; see Table 2) ad libitum. Micronutrient content was controlled between the two diets. All food was placed in metal food hoppers and weighed every other day. In all experiments, mice were given CD or HFD during the CSDS paradigm.

Social Interaction Test

The social interaction test (SIT), a behavioral assay assessing social avoidance, is used as a readout to validate the CSDS paradigm. Each SIT was performed 24 hours after the last defeat session, with an additional test performed after the fluoxetine treatment. Briefly, the SIT is composed of two-sessions. In the first session, a mouse is allowed to explore an open field arena (40 cm × 40 cm) for 2.5 minutes. Along one side of the arena is a wire mesh cage that remains empty during the first trial (i.e., no social target present). This mouse is then removed, and a novel CD-1 mouse is placed into the wire mesh cage (i.e., social target present). The test mouse is then brought back into the arena and the amount of time it spends in the “interaction zone” (8 cm wide

corridor surrounding the cage), as well as the time spent in the "corners" farthest from the mesh cage, are measured during the second 2.5-minute trial (social target present). Socially defeated mice explore the interaction zone significantly less when another mouse is present, spending more time in the corners. Interaction ratios below 1.0 indicate social avoidance and susceptibility to stress (Berton and Nestler, 2006; Warren et al., 2013).

RT-qPCR

Mice were sacrificed 24 hours after the last day of sucrose preference. Bilateral punches were taken from the NAc (14 gauge) and VTA (15 gauge) and stored at -80°C until use. RNA was isolated using RNEasy Micro kits (QIAGEN) and cDNA was created from these samples using iScript cDNA synthesis kits (Bio-Rad). Quantitative real-time reverse transcription-PCRs (qPCRs) were performed in triplicate using 96-well PCR plates and SYBR Green MasterMix (Thermo Fisher) with a Bio-Rad CTX Connect 384 according to manufacturer's instructions. Threshold cycle [C(t)] values were measured using the supplied software and analyzed with the ΔΔC(t) method, as described previously (LaPlant et al., 2010; Vialou et al., 2010). Primer sequences for *Akt*, *Erk1*, *Erk2*, *Ccl2*, *NfκB*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are listed in Table 1.

RNAscope

RNA in situ hybridization (ISH) was performed using RNAscope V2 Assay (Advanced Cell Diagnostics) for *Akt* (Mm-Akt1-C1; Cat No. 455171), DRD2 (Mm-Drd1a-C2; Cat No. 406491), and DRD1 (Mm-Drd2-C3; Cat No. 406501) probes. Protocol was performed per manufacturer's instructions. Animals were perfused with 4% paraformaldehyde (PFA) and the

brains were left overnight in PFA. Brains were stored in cryoprotectant at -20°C until sliced in a vibratome (Leica VT1000S) at 40 µm. Nucleus accumbens (NAc) slices were mounted to Super Frost Plus slides (Fisher Scientific; Cat No. 12-550-15). Slides were postfixed in 4% PFD in 1x PBS for 1 hour at 4°C. The slides were dehydrated in 50, 70, and 100% ethanol, and subsequently dried at room temperature for 5 minutes; an ImmEdge Hydrophobic Barrier PAPpen (Vector Laboratories; Cat No. H-4000) was used to draw on the slides around the brain sections. The slides were then treated with protease solution at room temperature for 30 minutes, followed by washing in 1x PBS. Probes were then applied and incubated at 40°C for 2 hours in the HybEZ oven. Slides were incubated with amplifier probes (AMP1, 40°C for 30 minutes; AMP2, 40°C for 30 minutes; AMP3, 40 °C for 15 minutes) and then incubated with fluorescently labeled probes Opal 520 nm, Opal 560 nm and Opal 690 nm for *Akt*, D1, and D2, respectively. Sections were incubated with DAPI for 10 minutes. After washing, slides were cover slipped using ProLong Diamond Antifade Mountant (Thermo Fisher; P36961). Images were taken within 1 week of staining on a Zeiss LSM780 confocal microscope. Confocal images were acquired bilaterally with a 20× objective. Quantifications were performed using ImageJ software (National Institutes of Health, Bethesda, MD). Image analyses were performed in ImageJ using custom-built procedures. Nuclei were first segmented by a binarization of the DAPI signal. The fluorescence was measured inside the nuclei masks to discriminate fluorescence levels in the nucleus for Drd1 and Drd2 probes. Cell type classification used user-defined thresholds of fluorescence intensity. Puncta of Akt signal were quantified within each nucleus using the ComDet v5 plug-in in ImageJ.

Magnetic Cell Sorting

Magnetic isolation of microglia was perform as described (Holt et al., 2019). A total of 32 male mice were sacrificed across four days, with the groups counterbalanced daily. All in-vivo isolations were performed at the same time daily to prevent changes due to diurnal hormonal fluctuations (Mazzoccoli et al., 2011). Brains were rapidly removed, and 14-gauge brain punches were taken for the NAc and left on ice 1xPBS. Samples were physically disrupted with scissors and digested in papain for 10 minutes at 37°C. After incubation, the sample is titrated and filtered to remove undissolved tissue. Suspended cells are then incubated with Cd11b+ microbeads (Miltenyi Biotec; Cat No. 130-049-601) for 15 minutes at 4°C to tag microglia. Cells are washed, centrifuged, resuspended, and ran through the magnetic columns. The flowthrough is then collected for further processing. The column is removed from the magnetic rack and placed for elution into a 15 mL centrifuge tube. After centrifugation, the supernatant is discarded, and the pellet is suspended in 300 µL of Trizol, and flash-frozen on dry ice. Further processing of the microglia was completed as described in the RT-qPCR section above.

Viral-mediated gene transfer

For stereotaxic delivery of the viruses, mice were anesthetized with a ketamine/xylazine cocktail (80/10 mg/kg; intraperitoneal). The mice were infused with the virus using a Hamilton 33-gauge bilateral microinjection needle (0.5 µl per side at a rate of 0.1 µl/ min.). Mice received either the control adeno-associated virus (AAV5-U6- scrambled, or the short-hairpin RNA for functional downregulation of *Akt* (AAV5-U6-shRNA-*Akt*1; shAAV-252433) at a titer of $1x10^{12}$. NAc was targeted using a 10° angle to avoid the sinus system (anteroposterior 1.6; lateral +1.8; dorsoventral –4.4 in the mm from bregma).

Statistical analyses

Data were analyzed using GraphPad Prism (version 9) software. When appropriate, two-way ANOVAs or Student's t-tests were used to determine statistical significance of pre-planned comparisons. Data are expressed as the mean ± SEM, with statistical significance set at $p<0.05$.

Results

Characterization of simultaneous chronic social defeat and high-fat diet exposure.

Experimental Design. After habituation, adolescent mice (PD35) were randomly assigned for synchronous exposure to chronic social defeat stress (CSDS) and high-fat diet (HFD). Non-stressed controls (CON) received either a control diet (CD; n=8) or high-fat diet (HFD; n=8) similar to physically stressed (PS) mice (n=8/group) for 10 days and then subsequently tested in the social interaction test (SIT) 24 hours after the last defeat (PD45; Fig. 20*A*)

Body Weight. Body weight was measured every other day throughout the defeat protocol. The final body weight was subtracted from the initial body weight and averaged across groups (Fig. 20*B*). A two-way repeated-measures ANOVA showed that mice lost weight as a factor of stress ($F_{(1,28)}$= 5.494; p<0.05).

Adjusted Caloric Intake. Caloric intake was calculated from food consumption divided by respective body weight (Fig. 20*C*). A three-way multivariate-ANOVA (MANOVA) revealed significant changes in adjusted caloric intake as a factor of time ($F_{(3, 64)}$=8.554; p<0.0001), diet ($F_{(1,64)}$=27.15; p<0.0001), and time x diet interaction ($F_{(3,64)}$=5.816; p=0.0014), with a trending interaction between stress and diet ($F_{(1,64)}$=3.826; p=0.0548). Post-hoc analysis revealed that CON+HFD- (p<0.05) and PS+HFD-exposed mice (p<0.05) consumed more calories during the second day of defeat+diet exposure compared to the CON+CD (p<0.05), whereas the PS+CD-exposed mice consumed significantly fewer calories (p<0.05). There were no significant differences between the groups during the last day of measurement (p>0.05).

Social Interaction. The effect of simultaneous CSDS and HFD exposure on social interaction was examined and represented by an interaction ratio (IR; Fig. 20*D*). A two-way ANOVA revealed that social IR differed between groups as a factor of stress ($F_{(1,29)}$=39.34;

$p<0.0001$), where both the PS+CD- and PS+HFD-exposed mice both had significantly lower IRs compared to the CON+CD-exposed group ($p<0.05$). Though the social IR did not differ significantly between stress groups, all the HFD-exposed mice showed a susceptible phenotype, with no resilient subjects (Fig. 20*E*).

Changes in gene expression of secondary messenger systems within limbic regions after simultaneous CSDS and HFD exposure.

Quantitative rt-PCR was used to assess gene expression changes between mice exposed to simultaneous CSDS and HFD. Two-way ANOVA demonstrated that within the NAc, *Akt* expression was dysregulated as factor of both stress ($F_{(1,28)}=22.67$; $p<0.0001$) and diet ($F_{(1,28)}=17.29$; $p=0.0003$; Fig. 21*A*). Post-hoc analysis revealed that both PS+CD- ($p<0.01$) and PS+HFD-exposed mice ($p<0.05$) had significantly increased *Akt* expression compared to the CON+CD-exposed group. The PS+HFD- also had significantly higher *Akt* mRNA levels than the PS+CD-exposed mice ($p<0.05$). Expression of *Gsk3β* was also influenced by diet (Fig. 21*B*; $F_{(1,28)}=5.634$; $p=0.0247$) as post-hoc analysis showed that both the CON+HFD- ($p<0.01$) and the PS+HFD- ($p<0.05$) exhibited a decrease in expression compared to the CON+CD-exposed mice. Expression of *Erk1* was influenced by diet ($F_{(1,28)}=9.282$; $p=0.005$) and stress ($F_{(1,28)}=5.674$; $p=0.0243$; Fig. 21*C*). Post-hoc analysis indicated that the PS+HFD-exposed mice had significantly higher *Erk1* levels compared to all the other groups ($p<0.05$). There were no significant differences in *Erk2* mRNA levels observed in the NAc regardless of experimental conditions (Fig. 21*D*; $p>0.05$).

Within the VTA, *Akt* expression was affected by both diet ($F_{(1,28)}=5.499$; $p=0.0264$) and stress ($F_{(1,28)}=103.5$; $p<0.0001$; Fig. 21*E*). Post-hoc analysis showed that both the PS+CD- and

PS+HFD- had significantly reduced expression of *Akt* compared to the CON+CD-exposed mice ($p<0.001$). *Gsk3β* expression was significantly altered by diet ($F_{(1,28)}=10.20$; $p=0.0035$) and stress ($F_{(1,28)}=17.2$; $p=0.0003$; Fig. 21*F*). Post-hoc analysis illustrated that the PS+HFD- had significantly lower expression of *Gsk3β* compared to the CON+HFD-exposed mice ($p<0.05$). *Erk1* signaling was also evaluated and found to be differentially regulated as a factor of stress ($F_{(1,28)}=18.39$; $p=0.0002$; Fig. 21*G*), with post-hoc analysis demonstrating that the PS+HFD- had significantly increased *Erk1* levels compared to CON-HFD-exposed mice ($p<0.01$). Similarly, *Erk2* gene expression was influenced by stress ($F_{(1,28)}=17.83$; $p=0.0002$; Fig. 21*H*) with post-hoc analysis revealing that the PS+HFD- had significantly higher *Erk2* expression than the CON+HFD-exposed mice ($p<0.05$).

Cell-type expression of Akt within the nucleus accumbens.

RNAscope in-situ hybridization assays were performed to determine whether changes in *Akt* expression as seen in Fig 22*A* were co-localized with D2 (Fig. 23; red) and D1 (yellow) receptors in individual DAPI (blue) labeled cells. Columns represent the different probes (left to right; *Akt*, D2, D1, DAPI, and Merged). The rows represent different groups (top to bottom; CON+CD, CON+HFD, PS+CD and PS+HFD). Representative images from sections from 1 mouse in each group are shown.

Quantification of Akt expression of with D1 and D2 expressing cells.

To determine whether changes in *Akt* expression were localized within D1 or D2-containing cells, RNAscope assay images were run through a custom ImageJ program to quantify the *Akt* puncta within DAPI labeled cells (n=2/group; 4 images per section per group). The relative

abundance and cell type localization was calculated and presented as puncta/cell. As seen in Fig. 23*A*, there was a significant reduction in *Akt* abundance in D1 neurons as a factor of diet ($F_{(1,4)}$=39.94; p=0.0032) and stress ($F_{(1,4)}$=11.02; p=0.0294). Post-hoc analysis showed that CON+HFD- (p<0.01) and PS+HFD- (p<0.05) were significantly lower than the CON+CD-exposed group. Similarly, within D2 neurons, *Akt* was similarly dysregulated by diet ($F_{(1,4)}$=77.20; p=0.0009) and by an interaction between diet and stress ($F_{(1,4)}$=9.570; p=0.0365; Fig. 23*B*). All mice exposed to HFD showed a decrease in *Akt* expression (p<0.05), while the PS+HFD- had lower *Akt* expression than the PS+CD-exposed mice as well (p<0.05).

Assessing the involvement of microglia in the deficits induced by early-life CSDS and HFD exposure.

To further delineate whether the CSDS+HFD-induced effect on *Akt* expression was cell-type specific, microglia were sorted and processed for RNA. qPCR revealed that *Akt* gene expression was influenced by stress ($F_{(1,24)}$=4.513; p=0.0441; Fig. 24*A*), but no changes were observed in *Ccl2* or *NfκB* (p>0.05; Fig. 24*B-C*).

Viral manipulation of Akt within the NAc in CSDS+HFD-exposed adolescent mice.

After habituation, adolescent mice were microinfused with AAV-scrambled (n=20; Fig. 25*A*) or AAV-shRNA-*Akt* (n=20). After surgery, the mice were allowed to rest for 2 weeks and then randomly assigned for exposure to CSDS and either CD or HFD (n=5/group). Social interaction was measured 24 hours after the last defeat. Three-way MANOVA showed that interaction ratio was influenced by stress ($F_{(1,25)}$=25.99; p<0.0001) and a three-way interaction between Virus x Stress x Diet ($F_{(1,25)}$=5.170; p=0.0318; Fig. 25*B*). Post-hoc analysis revealed that

within the scrambled, control virus-exposed groups, both groups, the PS+CD and PS+HFD-exposed mice showed significant reductions in interaction compared to CON+CD-exposed mice. Furthermore, in the shRNA-*Akt* group, only the PS+HFD- showed reductions in interaction ratios as compared to the CON+CD-exposed mice ($p<0.05$). To confirm that the virus did in fact reduce *Akt* mRNA levels, a separate group of mice were infused with virus (n=5/group) and were then sacrificed for biochemistry assessment. As can be seen in Fig. 25*C*, *t*-test showed that shRNA virus significantly reduced *Akt* levels ($t_{(8)}=2.588$; $p<0.05$).

Discussion

Stress and western style-diets, such as high-fat/high-carbohydrate diets (HFDs), are major insults to the body, especially during the sensitive period of adolescence (Neustadt, 2006; Yaribeygi et al., 2017). There are a multitude of biological systems (i.e., neurobiological, inflammatory, microbiota) that have been implicated in the pathophysiology of the comorbidity between diet- and stress-induced disorders, which makes it highly unlikely that a single target would be responsible for the totality of overlapping symptomologies. However, targets like the protein kinase B (*Akt*), which is found ubiquitously in the brain and in many cell types, have been implicated in both stress- (Krishnan et al., 2008; Parise et al., 2021) and metabolic-induced responses (Dutheil et al., 2016; Lawan et al., 2018). In this chapter, I characterized the involvement of *Akt* within the NAc using bulk tissue and demonstrate visualization of *Akt*, specifically in D1- and D2-containing medium spiny neurons (MSNs), and within microglia. Furthermore, I evaluated whether viral-mediated manipulation of *Akt* could induce a resilient phenotype to chronic social defeat stress (CSDS) that could be blocked by HFD exposure. Overall, my findings show a significant dysregulation of *Akt* after simultaneous CSDS and HFD exposure, and that this profile of dysregulation differs between cell-types.

The few studies that have utilized HFD concomitantly with CSDS have done so using adult rodents (Coccurello et al., 2018). To bridge this gap, I exposed adolescent (PD35) mice to simultaneous CSDS (also referred to as physical stress; PS) and control (CD) or HFD diet. Body weight and food intake were measured across the 10 days of PS exposure yielding an effect of stress on body weight, but no effect of HFD. Previous studies have shown that exposure to PS causes a reduction in weight gain (Warren et al., 2013) thus it is likely that at least one more week of HFD exposure may be needed to observe an obesogenic phenotype, given the results I presented

in Chapter 2. (Fig. 3*B*, 6*C*). Adjusted caloric intake was determined from food intake and results indicated that the HFD-exposed mice consumed more calories on day 2 compared to their CD-fed counterparts. This novelty-induced hyperphagia, which was observed in all other HFD-exposed groups, was diminished by day 4. As a confirmation of the effectiveness of the defeat and to evaluate whether HFD exposure during CSDS elicited depressive-like behaviors, the mice were assessed for social interaction. I found that the PS-exposed mice showed significant avoidance, but there were no significant differences as a factor of diet, indicating that the presence of an appetitive stimulus during the defeat paradigm does not alleviate the effects of stress on social interaction. In fact, the mice were separated into susceptible and resilient categories based on their interaction ratios, and it was found that all the PS+HFD-exposed were susceptible to stress, whereas the PS+CD-exposed mice had a more typical 3:1 (susceptible: resilient) split (Golden et al., 2011). Though this effect would require more power for confirmation, other studies have also found that HFD exposure can lead to susceptibility to stress (Ip et al., 2019; Kalyan-Masih et al., 2016). These data led me to conclude that there may exist neurobiological differences between the PS+CD- and PS+HFD-exposed mice, which could account for the increased susceptibility to stress, despite the lack of behavioral differences.

To confirm this hypothesis, I extracted RNA from the NAc and the VTA of adolescent mice exposed to simultaneous CSDS+HFD. Second messengers such as *Akt*, *Gsk3*β, and *Erk1/2* have been heavily implicated within these brain regions in reward response as well as antidepressant efficacy (Crofton et al., 2015b; Iñiguez et al., 2010a, 2014a; Krishnan et al., 2008; Russo et al., 2007; Zanos and Gould, 2018). The connections between these kinases are depicted in Appendix B3. I discovered that within the NAc, *Akt* and *Erk1* expression were influenced by both diet and stress in that the PS+HFD-exposed groups demonstrated significant increases in gene

expression. These results are fascinating as it has previously been shown that an increase in *Akt* or *Erk* signaling within the VTA is associated with a depressive-like phenotype (Iñiguez et al., 2014a; Krishnan et al., 2008), indicating that early-life stress and diet may be creating synergistic deficits. In addition, *Gsk3*β expression was reduced as a result of HFD exposure, which is interesting since *Gsk3*β reduction in the NAc has also been associated with exposure to drugs of abuse such as alcohol or methamphetamine (Rodd et al., 2008; Xing et al., 2015). There were no significant changes observed in *Erk2* expression levels after stress, which is unusual given that other studies have reported an increase in *Erk2* in adolescent mice after CSDS (Warren et al., 2014). Within the VTA, *Akt* levels decreased as a factor of stress, findings in accordance with the literature (Krishnan et al., 2008), with no effect of HFD exposure. Surprisingly, though *Erk1* and *Erk2* were increased as a factor of stress as previously described (Iñiguez et al., 2010a), the PS+HFD-exposed mice had the highest levels of *Erk1/2* expression compared to both CON+CD and CON+HFD counterparts, indicating a compounding deficit. The expression of *Gsk3*β was altered by both stress and diet exposure, as the PS+HFD-exposed mice showed a substantial reduction in gene activity. Similarly to the NAc, *Gsk3*β activity in the VTA is reduced after drug exposure, specifically ketamine abuse self-administration (Huang et al., 2015). These data suggest that simultaneous CSDS+HFD exposure during adolescence leads to major changes in key second messenger systems resulting in compounding dysregulation in *Akt* and *Erk* expression within the NAc. Given these findings, I chose to pursue *Akt* due to its level of dysregulation and involvement in stress susceptibility, however, the other targets provide a novel avenue to explore in future research. The summary of these results can be seen in Appendix A2.

The NAc is primarily composed of D1- or D2-receptor containing MSNs (Kronman et al., 2021) and if the effect of *Akt* is present in neurons, it would most likely be within one of these

subsets. To this end, brains from adolescent mice exposed to simultaneous CSDS and HFD were run through the RNAscope assay for in-situ hybridization visualization of *Akt* mRNA and colocalized with either D1 or D2 receptor-containing neurons. Interestingly, *Akt* expression was observed ubiquitously throughout the section, which is unusual given that *Akt* is not confined to $DAPI^+$ cells, a potential limitation to this experiment. Automated analysis was performed to measure the abundance of *Akt* within $DAPI^+$ $D1^+$ or $D2^+$ cells. Surprisingly, *Akt* levels were decreased as a factor of diet in that all HFD-exposed mice showed lower abundance of *Akt* regardless of D1 or D2 receptor subtype. This unexpected result indicated that the effect observed in bulk tissue was not a D1- or D2-mediated effect. Given some evidence that *Akt* is predominately expressed within microglia (Zhang et al., 2014), I hypothesized that *Akt* may be upregulated in microglia.

To test this hypothesis, the NAc of adolescent mice exposed to simultaneous CSDS and HFD were extracted, and microglial cells were magnetically isolated. qPCR was performed on *Akt* and chemokine (c-c motif) ligand 2 (*Ccl2*), a marker of microglial activation. I hypothesized that microglia would have upregulated *Akt* due to the combined effects of stress and diet and that there would be an increase in activated microglia, as studies have found that HFD induces microgliosis within the hypothalamus (André et al., 2017; Carey et al., 2019; Valdearcos et al., 2017), with limbic inflammation accounting for some of the observed deficits (Décarie-Spain et al., 2018; Menard et al., 2017). As expected, there was an increase in expression of *Akt* within the microglia of the NAc in response to stress as previously shown (Stein et al., 2017). Increases in *Akt* expression within microglia has been associated with proinflammatory activity (Cianciulli et al., 2016), so it is surprising that *Ccl2* and *NfκB*, a downstream target of *Akt* that regulates inflammatory pathway, were unchanged. Overall, this data suggests that the effect observed in

bulk tissue was emulated in microglia, however, the expected increase in activated microglia and inflammatory factors was not noted. This is most likely due to the short duration of the diet exposure, as some studies have shown that it takes four months of HFD to induce inflammatory microglia activation (Valdearcos et al., 2014). These results confirm the role of microglia in the compounding deficits observed after simultaneous CSDS and HFD exposure, though more studies are needed to characterize what exactly the changes in *Akt* levels mean for the activity of microglia.

Studies have demonstrated that viral-mediated downregulation of *Akt* can inhibit the effects of CSDS in adolescent mice (Parise et al., 2021). Given that *Akt* is upregulated within bulk tissue and microglia in response to stress and HFD exposure, I hypothesized that downregulation of *Akt* would be unable to induce a resilient phenotype in the PS+HFD-exposed mice. To this end, adolescent mice were bilaterally microinfused with a short hairpin RNA (shRNA) intended to decrease *Akt* mRNA levels. qPCR analysis in a separate subgroup of microinfused mice showed that the shRNA-*Akt* virus did in fact reduce *Akt* expression. An adeno-associated virus (AAV) serotype 5 was used, which is known to infect astrocytes, neurons, oligodendrocytes, and microglia in striatum (Aschauer et al., 2013). After a 2 week incubation period, mice were exposed to simultaneous CSDS and HFD and tested in the social interaction test (SIT) 24 hours after the last defeat. As expected, PS-exposed mice infected with a control scrambled virus exhibited normal avoidant phenotype regardless of diet exposure. Interestingly, exposure to the shRNA virus blocked the effect of CSDS in the PS+CD-exposed mice. Surprisingly, the PS+HFD-exposed mice did not share this resilient phenotype and exhibited significant social avoidance. These results suggest that HFD exposure blocks the resilience that has been observed after *Akt* downregulation and that the susceptibility induced by HFD may be mediated, at least in part, through *Akt* activity.

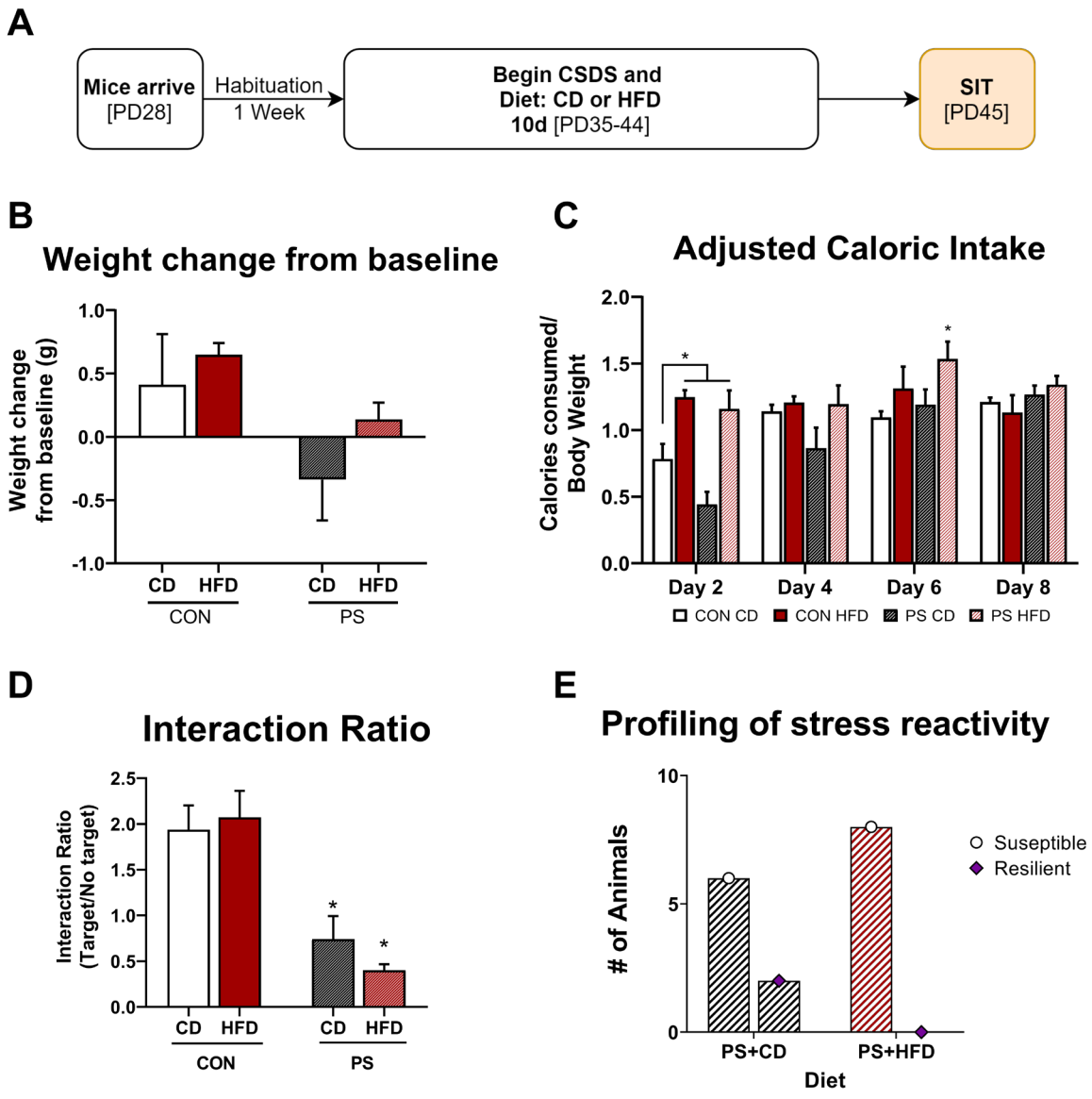
It is important to note that there are several limitations to this study. First, no female mice were included. There is evidence that females are at an increased risk of developing obesity after a stressful event and are more likely to suffer from depression compared to males (Mannan et al., 2016). The stress paradigm utilized here is not amenable to the use of female mice, as there is no validated method of successfully encouraging males to aggressively charge female mice. Although some approaches have involved the use of more aggressive strains of female mice, optogenetic manipulations in transgenic mice, and male pheromones to incite males to show aggression toward a female (Harris et al., 2018; Newman et al., 2019; Takahashi et al., 2017), these newer models have not yet been fully characterized and validated. In addition, long-term measurements of weight were not taken to examine whether this paradigm also resulted in rapid obesogenic changes. Furthermore, western blot analysis of these second messenger targets is of crucial importance to understand whether the effects at the mRNA level are translated at the level of proteins. Finally, the expression of *Akt* in D1- and D2-specific MSNs should be validated using immunohistochemistry. This would further confirm that the effects noted at the level of gene expression translate to the expression of gene-specific proteins.

In this chapter, I aimed to biochemically characterize a concomitant/simultaneous version of CSDS and HFD exposure. My findings implicate dysregulation of second messenger system within limbic regions such as the NAc and the VTA. Expression in *Akt*, *Erk1/2*, and *Gsk3β* levels were dysregulated after simultaneous CSDS and HFD exposure. Compounding deficits were observed in *Akt* and *Erk1* mRNA levels within the NAc. I further demonstrate that the increase in *Akt* mRNA levels in bulk tissue was not observed when quantified via in-situ hybridization. The abundance of *Akt* was reduced in both D1- and D2-containing neurons as a result of HFD exposure. Interestingly, however, *Akt* levels in microglia were also upregulated in the PS+HFD-exposed

groups, suggesting that stress and diet interact to produce synergistic deficits in *Akt* expression and microglia function. Finally, the resulting resilient phenotype induced by viral-mediated downregulation of *Akt* within the NAc was inhibited by HFD exposure, suggesting that HFD may act through *Akt* to mediate stress susceptibility. Overall, these data highlight the need to further explore the complex interactions between early-life stress and western-style diet and how the dysfunction of secondary messenger systems in various cell-types within the mesolimbic circuit are responsible for metabolic and mood-associated comorbidities.

Figure

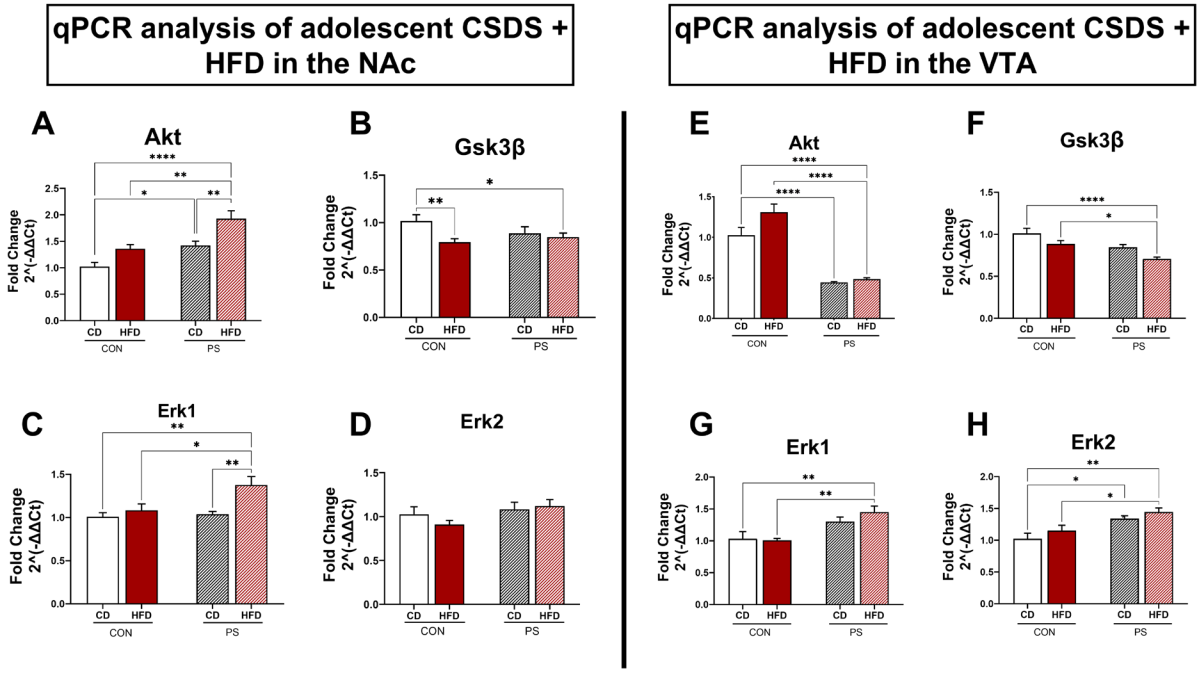
Figure 20. Characterization of simultaneous chronic social defeat stress and high-fat diet exposure.



A) Adolescent mice (PD35) were randomly selected for synchronous exposure to chronic social defeat stress (CSDS) and high fat-diet (HFD). Non-stressed controls (CON) received either control

diet (CD; n=8) or high-fat diet (HFD; n=8), similar to physically stressed (PS) mice (n=8/group) for 10 days and subsequently tested in the social interaction test 24 hours after the last defeat (PD45) **B)** Exposure to stress led to decreases in weight from baseline ($p<0.05$) with no effect of diet ($p>0.05$). **C)** Adjusted caloric intake was influenced by time ($p<0.05$) and diet ($p<0.05$). During the second day of defeat, HFD-exposed mice consumed more calories compared to their CON-CD counterparts ($p<0.05$). Alternatively, PS-CD mice consumed fewer calories ($p<0.05$). This effect was lost by the last measurement day, as there were no differences between the groups ($p>0.05$). **D)** Mice exposed to stress, regardless of diet, showed a decrease in interaction ratio ($p<0.05$). **E)** Though there were no significant differences in interaction ratio between the stressed groups, PS-HFD mice were all behaviorally susceptible to stress, whereas PS-CD had a typical dichotomous split. Data is shown as SEM, $^{*}p<0.05$.

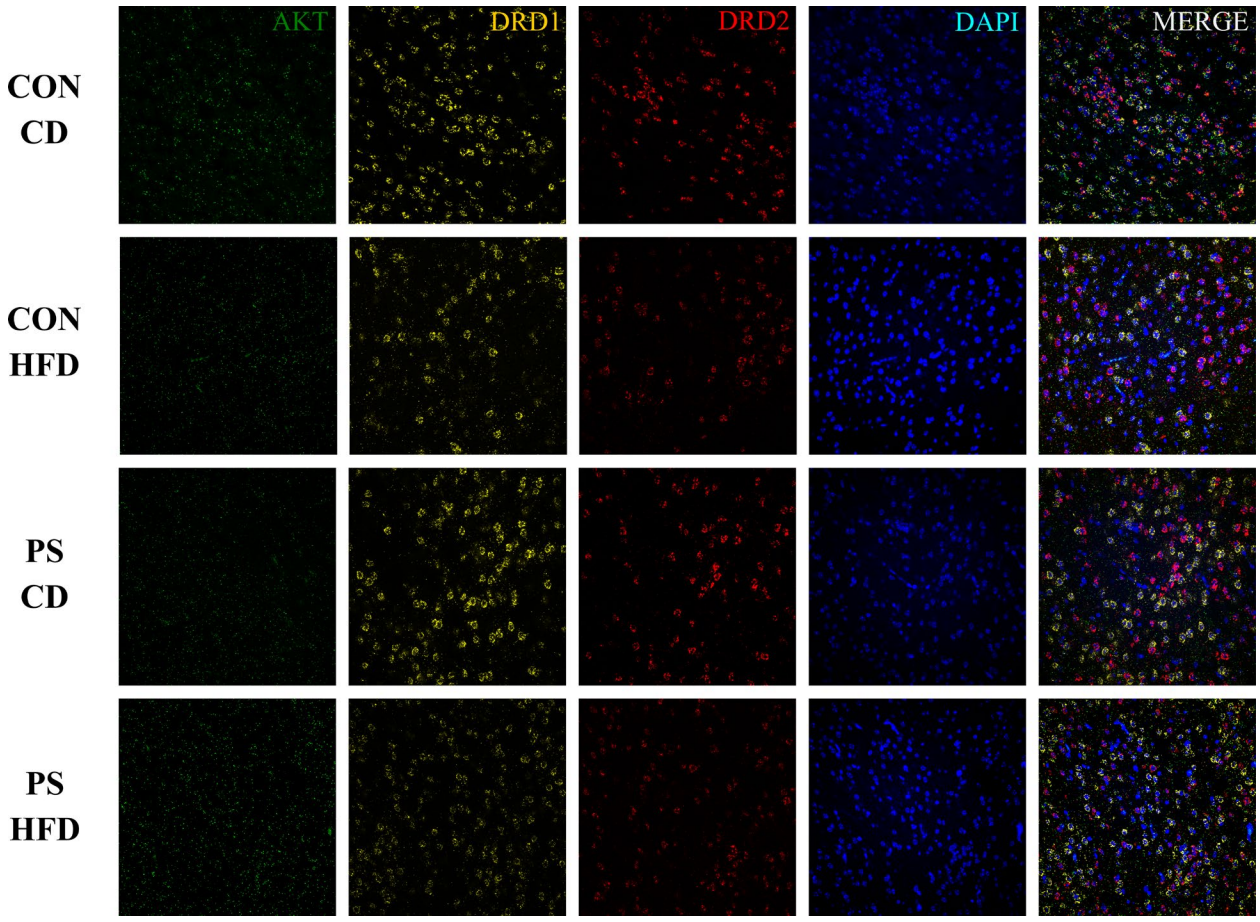
Figure 21. Changes in gene expression of secondary messenger systems within limbic regions after simultaneous CSDS + HFD exposure.



Brain punches were collected 24 hours after the social interaction test. (Fig. 22) **A)** Expression of *Akt* was influenced by stress ($p<0.0001$) and diet ($p<0.001$). Post-hoc analysis revealed that the concurrent effect of PS and HFD caused a significant increase in *Akt* expression ($p<0.05$). **B)** Mice exposed to HFD showed a significant reduction in *Gsk3β* ($p<0.05$). **C)** *Erk1* signaling was dysregulated by both diet ($p<0.05$) and stress ($p<0.05$), and mice exposed to PS+HFD had the highest level of *Erk1* expression compared to any other group ($p<0.05$). **D)** There were no changes in *Erk2* signaling in response to stress or diet in the NAc ($p>0.05$). **E)** Within the VTA, *Akt* gene expression was altered by both diet ($p<0.05$) and stress ($p<0.0001$), where all groups of mice exposed to PS showed significant reduction in *Akt* mRNA ($p<0.001$). **F)** *Gsk3β* expression was altered by diet ($p<0.01$) and stress ($p<0.001$). Post-hoc analysis indicated that PS+HFD had

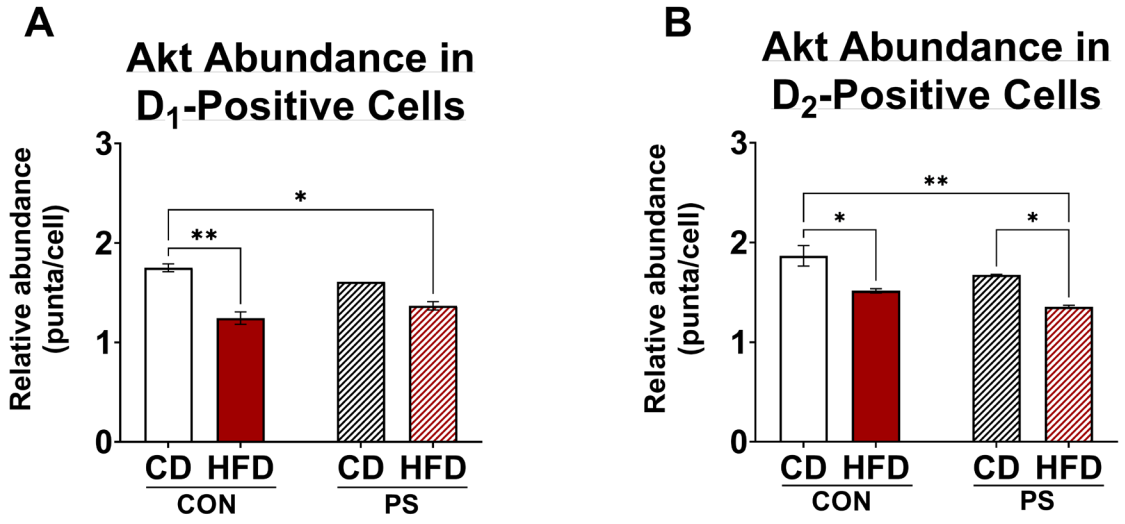
significantly lower expression of *Gsk3β* compared to both CON+CD ($p<0.05$) and CON+HFD ($p<0.05$).**G)** Stress altered the expression of both *Erk1* and **H)** *Erk2*. Only PS+HFD mice showed significant increases in *Erk1* expression, as revealed by post-hoc analysis ($p<0.01$). Both PS+CD ($p<0.05$) and PS+HFD ($p<0.01$) mice demonstrated an increase in *Erk2* levels.

Figure 22. Akt mRNA expression within the Nucleus Accumbens.



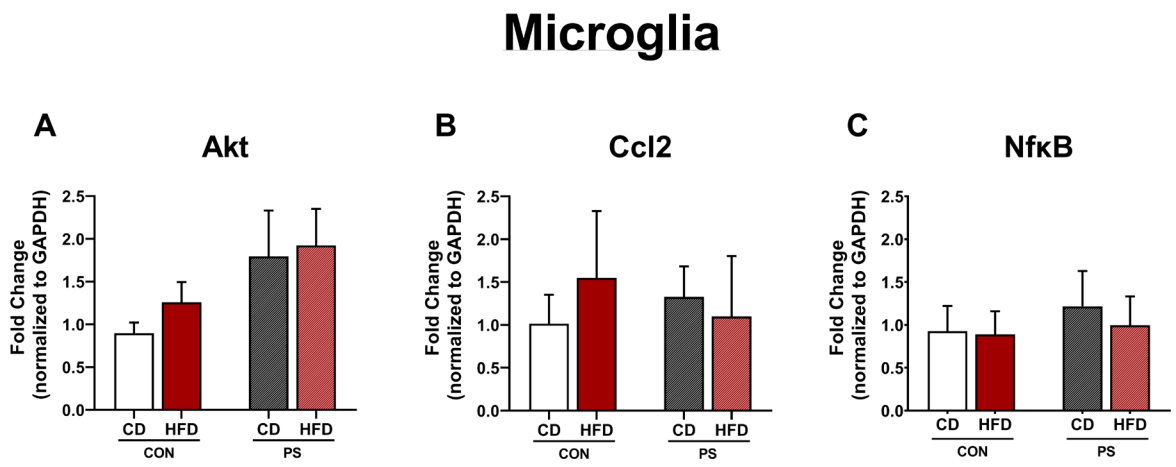
RNAscope *In-situ* hybridization assay was performed to evaluate whether the changes in *Akt* expression were seen within D1- (DRD1) or D2-(DRD2) expressing medium spiny neurons of the NAc. Columns represent the different probes (left to right; *Akt* (green), DRD2 (red), DRD1 (yellow), DAPI (blue), and merged). The rows represent different groups (top to bottom; CON+CD, CON+HFD, PS+CD and PS+HFD). Representative images from 4 sections from 4 mice/group are shown.

Figure 23. Quantification of Akt expression of with D1 and D2 expressing cells.



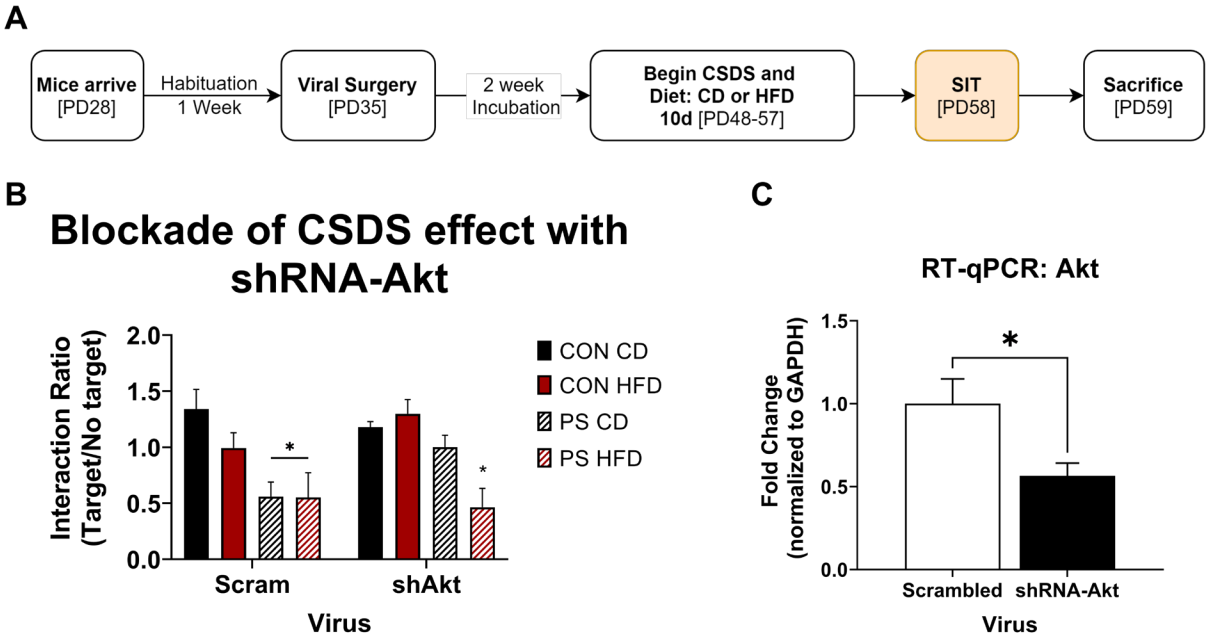
Quantification of *Akt* puncta signal from the RNAscope assay using custom ImageJ program allowed for assessment of abundance within D1- and D2- containing cells **A)** *Akt* signal was reduced in both CON+HFD and PS+HFD groups. **B)** In D2- containing cells, *Akt* signal was similarly reduced in CON+HFD ($p<0.05$) and PS+HFD ($p<0.01$). Moreover, PS+HFD showed a significant reduction compared to PS+CD ($p<0.05$).

Figure 24. Assessing the involvement of microglia in the deficits induced by early-life CSDS and HFD exposure.



Adolescent mice exposed to simultaneous CSDS and HFD were terminated and Cd11+ microglia cells were sorted from whole tissue and processed for RNA. **A)** qPCR showed that *Akt* was differentially regulated by stress ($p<0.05$). **B-C)** Neither *Ccl2* nor *NfκB* expression differed between groups ($p>0.05$).

Figure 25. Viral manipulation of Akt within the NAc in CSDS+HFD exposed adolescent mice.



A) Adolescent mice were surgerized after habituation with AAV-scrambled (Fig 25*A*; n=20) or AAV-shRNA-*Akt* (n=20), allowed to rest for 2 weeks, and randomly selected for exposure to CSDS and CD/HFD (n=5/group). Social interaction was measured 24 hours after the last defeat. **B)** In mice infected with scrambled virus, as expected, all animals exposed to PS- showed a decrease in interaction ratio when compared to CON+CD ($p<0.05$). On the other hand, PS+CD, mice infected with shRNA *Akt* showed normal levels of interaction, where PS+HFD mice still exhibited a social interaction deficit. **C)** Validation of virus was done in a separate group (n=5) and confirmed that the shRNA did in fact reduce levels of *Akt* mRNA ($p<0.05$).

CHAPTER VI
DISCUSSION AND FUTURE DIRECTIONS

Major depressive disorder (MDD) is a chronic and reoccurring condition that affects 350 million people (Das, 2016) and is one of the leading causes of disability globally (Friedrich, 2017). Because MDD is highly comorbid with various other disorders, including substance-use disorders, pain, anxiety, post-traumatic stress disorder (PTSD), and obesity, the treatment of depression has proven to be challenging (Mimura, 2001). Of particular interest to me is the problematic co-occurrence of MDD and obesity, especially given that obesity is directly related to a number of diet-related disorders such as diabetes, metabolic syndrome (MetS), and cardiovascular disease, which is the leading cause of mortality globally (Campayo et al., 2011; Global Burden of Disease Collaborative Network, 2014; Mathers and Loncar, 2006; Penninx et al., 2001; Skilton et al., 2007). Overweight or obese individuals make up 2.1 billion people around the world (Ng et al., 2014) and, unfortunately, a growing percentage of which include children and adolescents (Ogden et al., 2012). Impairments in metabolic health during adolescence increases the risk for early mortality (Neef et al., 2013), and similarly to adults, obesity during this developmental period is highly associated with MDD (Hammerton et al., 2014). This comorbidity is often referred to as the *metabolic-mood syndrome*, and though there is some dispute as to the precise clinical presentation of this phenomenon, there is mounting evidence delineating the overlapping dysfunctional systems that likely link these disorders (e.g., neurological, immunological, and microbiological; Appendix B1) (Chan et al., 2019; El Aidy et al., 2015; Lutter and Elmquist, 2009; Mansur et al., 2015).

Though there are a plethora of clinical studies that have demonstrated the connection between MDD and obesity (Lin et al., 2014; Luppino et al., 2010; Mannan et al., 2016), the comorbidity of these conditions is not easily modeled in preclinical research and oftentimes are studied in parallel. Diet-induced obesity models have long been used to emulate aspects of the MetS (Moreno-Fernández et al., 2018; Purkayastha and Cai, 2013). These studies have shown that prolonged consumption of western-style diets (WSD) that contain high amounts of fat and/or carbohydrates are associated with adiposity (i.e., obesity), insulin resistance, and reductions in hippocampal volume, with these reductions being implicated in the manifestation of mood dysregulation (Banfield et al., 2016; Freeman et al., 2014; Jacka et al., 2015; Singh et al., 2019). On the other hand, models of chronic stress have been used to precipitate depressive-like phenotypes (Krishnan and Nestler, 2011; Nestler and Hyman, 2010). Though the use of these models can independently assist in understanding of the mechanisms underlying this *metabolic-mood syndrome*, the combination of diet and stress is a far more clinically relevant paradigm, as both factors influence each other and are highly present during adolescence (Jacka et al., 2014; Kessler et al., 2007; Noble et al., 2019). A handful of studies have combined stress and high-fat and high-carbohydrate diet (HFD) exposure to induce weight gain, lipid dysregulation, and changes in mood-related behaviors (Chuang et al., 2010a, 2010b; Eudave et al., 2018); however, these studies often report contradicting findings. For example, and to further complicate matters, there is some literature that suggests that consumption of HFD may be protective against the effects of stress, though this study used 6 months – 1 year of diet exposure followed by stress during adulthood (Finger et al., 2011). The scarcity of studies using concomitant HFD and stress during early-life make it difficult to gain a full understanding of this comorbid conditions within the context of adolescence. Given the lack of basic research assessing the physiological and

neurobiological effects of concomitant HFD and stress exposure, in this dissertation I aimed to characterize a model for adolescent metabolic-mood syndrome using chronic and vicarious social defeat stress (VSDS), which are ethologically relevant stressors capable of inducing the core symptoms of depression (i.e., anhedonia, social avoidance, etc.) and post-traumatic stress disorder (Berton and Nestler, 2006; Golden et al., 2011; Sial et al., 2016; Warren et al., 2020). In conjunction with this early-life stress, I utilized a HFD comparable to the poor diet that is consumed in the west and globally (fat content 45% kcal from lard, carbohydrate content 35% kcal from sucrose) (Drewnowski and Popkin, 1997; Speakman, 2019). To this end, I explored whether early-life stress would potentiate the deleterious effects (i.e., weight gain, caloric intake, social interaction, reward sensitivity changes) of HFD exposure, or vice versa, during adolescence in male mice.

In Chapter 2, I first exposed adolescent (postnatal day [PD]35) mice to HFD for 5 weeks to investigate whether diet exposure alone would induce weight gain and/or changes in social interaction. The rapidly growing adolescent mice on HFD gained weight over time but this did not differ from the normal chow (NC)-exposed mice. There were no changes observed in the caloric intake when this was normalized to body weight, thus indicating that the feeding behavior of these mice was homeostatically regulated. The mice consumed more calories only during the first week, likely due to a novelty-induced hyperphagia with the HFD, but the effect was quickly brought down to baseline by week two. These findings are supported by current literature in that it has been consistently shown that developing an obesogenic phenotype in adult mice can take 8-20 weeks in order to see significant weight gain, despite the utilization of extremely high levels of fat (~60% kcal) in the diet (Moreno-Fernández et al., 2018; Speakman, 2019). These mice also exhibited

nominal changes in social interaction behavior, thus indicating that diet alone was not sufficient to disrupt their social interaction or induce physiological changes. To test whether HFD exposure would induce changes in reward reactivity, I tested these mice sensitivity to drugs of abuse such as morphine (0.5 and 1 mg/kg), as measured in the conditioned place preference test, a well-established behavioral assay used to assess drug reward (Alcantara et al., 2014; Carlezon, 2003). All the mice in the NC group that were conditioned to the 1 mg/kg morphine dose demonstrated a preference for the drug-paired compartment when compared to their saline-injected control mice. However, the preference for morphine in the HFD-exposed mice did not significantly differ from their saline-injected group. The mechanism underlying this apparent decreased for morphine preference is unknown. It is possible that the mice in the HFD condition are less sensitive to the rewarding effects of morphine, and this may indicate that higher doses are needed. Though speculative, this assumption is supported by the mice responsiveness to sucrose preference as the same neural pathways are engaged in the brain by natural and drug rewards (Naneix et al., 2017; Volkow et al., 2008, 2013). This finding of reduced preference to morphine is interesting and it is opposite to other studies have shown that HFD consumption during adolescence causes lasting changes within the DAergic pathways that sensitize the system to drugs of abuse such as amphetamine (Naneix et al., 2017). This discrepancy may be due to differences in how HFD interacts with the opioid system and their receptors as opposed to amphetamines, though more detailed studies will be needed. Nevertheless, these findings are in agreement with other studies demonstrating that exposure to WSD induces alterations within the limbic system that can influence motivation for natural and drug rewards and potentially induce drug seeking behavior. Delineating the mechanisms by which diet causes maladaptive modifications, whether through

changes in homeostatic signals or inflammatory response, will be of upmost importance to understand if there is a relation between poor diet and substance abuse.

I established that exposure to HFD alone had minimal effects on weight, social interaction, but impaired drug and natural reward-related behaviors (e.g., sucrose and morphine preference) when consumed during the period of adolescence. To understand if adding stress would alter any of these parameters, I exposed mice to diet prior to stress and, alternatively, stress prior to diet, to determine whether timing of the insults would have differential effects. I hypothesized that both experimental designs would result in behavioral deficits. Results indicate that mice exposed to HFD for two weeks prior to CSDS (or physical stress; PS) gained weight rapidly after the end of the defeat stress. Interestingly, the mice consumed more calories during the first week, but this effect, similarly to the non-stressed mice, was lost by week two. This demonstrates that the weight gain observed was not due to increased caloric intake, but instead to potential metabolic abnormalities in caloric efficiency leading to positive energy balance and increased adiposity (Chuang et al., 2010a; Moles et al., 2006). No changes in social interaction were observed in response to HFD pre-exposure before CSDS. All the PS-exposed mice demonstrated a reduction in interaction, indicating that HFD exposure does not block the detrimental effect of CSDS in adolescent mice. Unfortunately, I was unable to determine whether HFD pre-exposure could worsen social behavior given that CSDS may have induced a floor effect in social interaction. As briefly alluded to previously, I also tested for sucrose preference in the same group of mice at a wide range of concentrations (0.25%-10%). Mice exposed to PS have been shown to have reductions (i.e., anhedonia) in sucrose preference (Krishnan et al., 2007; Warren et al., 2013), so I hypothesized that HFD exposure would cause a further reduction in sucrose preference. I found

that HFD+PS- mice consumed significantly less sucrose compared to the NC+PS-exposed mice, confirming that concurrent stress and HFD exposure caused compounded deficits that are worse than stress alone. However, these results may be confounded, as sucrose is already present in the HFD, and the additional calories could potentially have affected their preference for the sweet substance. For this reason, all subsequent studies assessing preference for a sweet solution were conducted with saccharin, a non-caloric sweetener. To start building on the potential mechanism(s) underlying these behavioral effects, I probed for molecular markers known to respond to stress and rewards. Second messenger systems such as protein kinase B (*Akt*) and the extracellular signal-regulated kinase (*Erk*) are known to modulate both stress- and reward-related behavioral responsiveness within the nucleus accumbens (NAc) and ventral tegmental area (VTA) (Iñiguez et al., 2010a; Krishnan et al., 2007; Li et al., 2010). Here, I hypothesized that exposure to PS+HFD would lead to increases in *Akt* and decreases in *Erk* mRNA levels within the VTA, while I expected the opposite within the NAc. My hypothesis, however, was not confirmed, as *Akt* expression was downregulated, and *Erk1/2* were both upregulated, within the NAc. Within the VTA, *Erk1/2* were both significantly downregulated. These findings were surprising given that previous studies have shown that CSDS leads to decrease in *Akt* and increase in *Erk*2 within the VTA (Iñiguez et al., 2010a; Krishnan et al., 2008; Parise et al., 2021). However, viral-mediated upregulation of *Erk*2 within the VTA increases sucrose consumption despite studies showing *Erk*2 expression is decreased after CSDS, which is typically associated with decreases in sucrose preference (Iñiguez et al., 2010b). Although contradictive, it has been hypothesized that *Erk*2 overexpression can lead to sensitization of the reward system(s) independently of its ability to modulate depressive-like behaviors (Iñiguez et al., 2010b). It is also possible that these effects are confounded by the 12 days of sucrose preference testing and other behavioral testing that the mice were exposed to.

Furthermore, these results would need to be substantiated at the protein level with western blot analysis.

In my second pilot experiment, mice were exposed to CSDS before HFD diet. These mice exhibited similar rapid weight gain within one week of introduction to the HFD, and these effects were also independent of the caloric intake. This obesogenic-like phenotype was likely induced by a priming effect of stress on the peripheral system, exaggerating the effect of diet exposure. It is likely that inflammation mediated by stress is a major culprit in the development of this phenotype, as CSDS has been shown to increase proinflammatory cytokines (Menard et al., 2017), potentially worsening the inflammation caused by HFD exposure (Waise et al., 2015), and resulting in adiposity and insulin resistance, which are prevailing symptoms of many diet-induced disorders (Festa et al., 2001; Lee et al., 2011). Inflammation is also mediated by corticosterone (CORT; cortisol in humans), a hormone produced by the adrenal glands (Marieb and Hoehn, 2007; Taves et al., 2011). Given that adrenocorticotropic hormone regulates the production of CORT and is directly correlated with kidney weight in rodents (Akana et al., 1983), I assessed kidney weight in the PS+HFD-exposed mice. My expectation was that these mice would show higher weights because the increase in CORT levels would be correlated with higher kidney weights. Confirming this hypothesis, I found that the PS+HFD-exposed mice exhibited kidney hypertrophy, a phenomenon also observed in adult mice fed HFD, which is associated with kidney damage (Cheng et al., 2019). Furthermore, reward sensitivity was also altered in this group of mice, as the PS+HFD- consumed less saccharin (0.05 and 0.5% concentration) than the PS+NC-exposed mice. These results suggest that the stress before diet experimental design was sufficient to induce

reward deficits beyond stress alone, thus I chose to further investigate these effects following this paradigm in Chapters 3 & 4.

In Chapter 3, I aimed to elaborate on the stress-before-HFD exposure model described above, with some changes based on findings from my pilot experiments. First, I added non-stressed control groups, which are crucial for proper data analysis and interpretation. Second, I utilized a non-physical stressor to assess whether psychological or emotional stress (ES) could induce similar physiological and behavioral deficits as observed after PS exposure. Emotional stress is a major contributor to the behavioral abnormalities that can lead to physiological deficits (Escarfulleri et al., 2021). Additionally, the prevalence of psychological/emotional stress in early-life is about four times that of physical stress (Vachon et al., 2015), and is often prognostic of the development of psychiatric disorders in adulthood (Kessler et al., 2010). Therefore, I employed the vicarious social defeat stress (VSDS) paradigm, a modified version of the CSDS assay in which another C57 mouse solely "witnesses" a physical defeat. Exposure to VSDS has reliably shown to induce physiological and behavioral deficits similar to those observed in the PS-exposed mice on depressive-, anxiety-like and reward-related behaviors (Sial et al., 2016; Warren et al., 2013, 2020). My findings revealed that stressed mice, regardless of type of stress exposure (whether physical or emotional), demonstrated a rapid increase in body weight independent of caloric intake compared to control mice. This was fascinating because to my knowledge, this is the first study to demonstrate that exclusively vicariously experiencing a stressful/traumatic event was able to induce an obesogenic-like phenotype in adolescent mice. The similarities between the stress groups can potentially be explained by previous studies that demonstrated that exposure to ES induces similar proinflammatory states to that observed after PS exposure (Hodes et al., 2014). I

subsequently assessed social interaction 1 month after the start of HFD exposure to evaluate potential diet-induced changes in social behavior. Here I found that, similarly to the results in Chapter 2, there was no significant effect of HFD on the social avoidant phenotype demonstrated by the mice in the stress conditions. Lastly, the same group of mice were then exposed to a saccharin preference test. Both the ES- and PS-exposed mice exhibited a significant attenuation of saccharin preference compared to the mice in the CON+NC condition, indicating that stress, regardless of its nature, is capable of inducing deficits in reward regulation. These data thus far have demonstrated that early-life stress, when combined with a diet high in fats and carbohydrates, can induce rapid weight gain that is also associated with alterations in reward sensitivity. These findings, though speculative, imply that early-life stress may induce depressive episodes and when combined with highly palatable foods may cause enhancements in hedonic-seeking behaviors such as substance abuse later in life.

Most studies have utilized HFDs because of their resemblance to the western-style diet composition (Speakman, 2019), though fats have taken the brunt of the blame for the adiposity and overall health detriments associated with metabolic ailments (Guasch-Ferré et al., 2015; Sharma et al., 2007). Thus, the influence of overconsumption of diets high in carbohydrates is a global issue that is often overlooked (Avena et al., 2008; Noble et al., 2019; Vendruscolo et al., 2010). I hypothesized that diets that are low in fat but high in carbohydrates (LFD) would induce similar physiological and behavioral outcomes as seen after exposure to HFD. To this end, I exposed adolescent mice to VSDS followed by 4 weeks of LFD or HFD. I found that, contrary to my hypothesis, the LFD- did not gain weight as rapidly as the HFD-exposed mice after stress exposure. This finding demonstrated that the combination of high-fat with high-carbohydrates is

necessary to induce rapid weight gain, and that high-carbohydrate diets alone do not induce these effects, even after stress. This data is in support of many studies that have shown that dietary fat is a key contributor to obesity and metabolic dysfunction (Espitia-Bautista and Escobar, 2019; Luukkonen et al., 2018). I then examined whether 4 weeks of LFD exposure would influence social behavior after VSDS exposure. Like the HFD-exposed mice, exposure to LFD did not significantly influenced social interaction as all the stressed-exposed mice retained their social avoidant phenotype. Typically, chronic antidepressant treatment can be used to reverse the effects of VSDS (Sial et al., 2016; Warren et al., 2013). One of these antidepressants is the selective serotonin reuptake inhibitor, fluoxetine (FLX), which is the only FDA-approved antidepressants for depression during childhood and adolescence (Iñiguez et al., 2014a; U.S. Food and Drug Administration, 2014). Studies in adult mice have demonstrated that chronic (20 weeks) of HFD exposure can result in resistance to the antidepressant effects of FLX (Isingrini et al., 2010). I therefore hypothesized that mice exposed to either ES+HFD or PS+HFD would be resistant to the therapeutic effects of FLX, but that mice in the LFD condition would show normal reversal of stress-induced depressive phenotypes regardless of the type of stress experienced. I subjected the mice from the previous group to 21 days of forced FLX consumption in their drinking water. As expected, both the ES+HFD and the PS+HFD-exposed mice did not demonstrate a reversal of their avoidant phenotype, retaining the depressive-like social interaction behavior. Surprisingly, both the ES+LFD and PS+LFD-exposed groups did not exhibit any improvement, finding that is indicative of a loss of efficacy of FLX's therapeutic benefit. Together, these data indicate that despite the lack of physiological effects by LFD on body weight, it still induced neurobiological dysregulation resulting in the reduced efficacy of FLX, as seen with the HFD-exposed mice. The mechanism(s) underlying these effects is unknown. However, this phenomenon may be because

of FLX's mode of action being mediated not only through modification of serotonergic signaling but also via inflammatory pathways. Studies have shown that FLX reduces circulating cytokines in a rodent model of depression (Lu et al., 2017), and that the use of anti-inflammatory drugs such as aspirin can render FLX more efficacious (Brunello et al., 2006; Yang et al., 2014). Given that diets high in carbohydrates can also induce inflammation (Antunes et al., 2020; de Almeida-Souza et al., 2021), it is plausible that both LFD and HFD block the therapeutic effects of FLX by increasing systemic inflammation. The results of this chapter demonstrate that exposure to either ES or PS can cause rapid weight gain in response to HFD, but not to LFD. However, both LFD and HFD are sufficient to reduce the efficacy of the antidepressant FLX. Nevertheless, it must be noted that I only use a single behavioral output, the social interaction assay, and a more complete battery of behaviors assessing other depression-like behaviors would be needed to claim that the efficacy FLX was truly reduced and to what extent diet and stress induce depressive-like phenotypes.

In chapter 4, I sought to further characterize the neurobiological deficits I observed after exposure to the VSDS followed by HFD paradigm within the context of the gut-microbiome. In this experiment, I utilized a control diet (CD) that is very similar in composition to NC, but it is a more appropriate micronutrient-controlled to the HFD (Almeida-Suhett et al., 2019). This new CD ensures that any effects observed are not due to micronutrient content, which can have profound effects on behavior such as depressive-like symptoms in both humans and rodents (Levenson, 2006; Murray et al., 2016; Widmer et al., 1992). Given the evidence of the gut microbiome regulating neural and behavioral activity via hormonal and inflammatory alterations (Dinan et al., 2015), I sought to sequence the 16S bacterial DNA extracted from feces from animals exposed to

either ES or PS in conjunction with HFD. The 16S gene codes for the 16S ribosomal RNA, part of the small subunit of ribosomes that helps recruit mRNA for translation (Barandun et al., 2017). Given how this gene is highly conserved between bacteria, it allows for accurate taxonomic classification (Janda and Abbott, 2007). Results from the sequencing revealed that there were major alterations in bacterial composition from exposure to both stress and HFD. Being a potent modulator of microbiome structure, it is not surprising that HFD elicited a significant majority of the observed changes, with HFD exposure resulting in increased alpha diversity, or an overall increase in the various types of bacteria that are present in the gut. The significance of increased alpha diversity was fascinating as this has been also been reported in patients with active MDD (Jiang et al., 2015), indicating that an increase in bacterial diversity is not always a beneficial adaptation. Additionally, exposure to HFD induced an increase in the ratio of Firmicutes to Bacteroidetes, which is a marker for gut dysbiosis (i.e., an imbalance in microbial diversity and loss of beneficial bacteria) and overall poor gut health (Magne et al., 2020). This F:B ratio was highest in the group exposed to PS+HFD, further illustrating that the combination of these two insults leads to compounding deficits. I also examined the predicted altered metabolic pathways for the microbiome and found that over 50 of 340 analyzed pathways were dysregulated. Of these, the major alterations included amino acid metabolism, nucleotide synthesis, and carbohydrate metabolism. This is fascinating given that many of the dysregulated pathways, such as L-tryptophan and L-glutamate biosynthesis, are vital for neurotransmitter production and functioning, therefore indicating the influence that HFD exposure can have over both the bacterial composition and metabolic processes. Despite these observations, it is not known to what degree the production of neuroactive peptides from the gut microbiome influences neurobiology directly; however, there is ample evidence indicating that normal gut functioning is necessary for proper

nutrient balance and behavior (Foster et al., 2017; Magnusson et al., 2015; Meckel and Kiraly, 2019). To determine whether these alterations in microbiome composition and if the overall effect of PS and HFD led to changes in the stress hormone corticosterone (CORT) and the pro-inflammatory cytokine interleukin-6 (IL-6), I extracted serum from the same group of mice. I investigated CORT because its balanced regulation is crucial for proper functioning of systems that modulate mood under normal and stressful conditions (Warren et al., 2013; Zhao et al., 2008), as well as responses to diet (Guarnieri et al., 2012; Karatsoreos et al., 2010). I chose to investigate IL-6 because it is dysregulated in depressive disorder (Maes et al., 1995) and models of stress-induced depression (Hodes et al., 2016). In addition, this cytokine has been implicated in obesity and other metabolic syndromes (Eder et al., 2009), and reduction in visceral fat is linked to decreased levels of IL-6 (Wedell-Neergaard et al., 2019). I discovered that CORT was significantly increased in the PS+CD-exposed mice, and that IL-6 was also affected by stress alone. However, the PS+HFD-exposed mice exhibited the highest level of circulating IL-6. Together, these data, again, illustrate that the combination of early-life stress and HFD results in major alterations to the gut-microbiome as well as circulating stress hormones and cytokines. The increase in inflammation in the PS+HFD-exposed group could potentially explain the exaggerated weight gain and reductions in the antidepressant FLX's efficacy observed in Chapter 3; Figure 13*B* . How stress and diet specifically act together to influence the brain remains unclear.

In chapter 5, I tried to address the shortcoming that many of the assays done in the previous chapters were carried out in adulthood despite the experimental procedure beginning in adolescence, which is inherent to my experimental designs due to the short duration of adolescence in mice (~30 days) as well as the length of the diet/stress paradigm. To remedy this, I chose to

expose adolescent mice to HFD and the defeat stress concurrently. This truncated model allowed me to study phenotypical outcomes within the period of adolescence. I discovered that this abbreviated paradigm causes nominal short-term changes in physiology in that only the PS exposure was able to elicit weight loss by the end of the 10 days of stress exposure. Similarly to the previous data in Chapter 3 and Figure 10*B*, I observed no changes in interaction ratios between groups, with the PS+HFD-exposed group containing only stress-susceptible mice. To determine whether this increase in stress susceptibility was associated with changes in second messenger signaling, I measured *Akt* and *Erk* levels within the NAc. I found *Akt* and *Erk* were dysregulated within the NAc in response to simultaneous PS+HFD exposure. The compounding increase in *Akt* was most interesting, as it has been shown to increase susceptibility to stress (Krishnan et al., 2008). To determine in which cell type this effect may be occurring, I utilized RNAscope assay to visualize *Akt* abundance within populations of D1- and D2-containing medium spiny neurons of the NAc. Given the differential effects reported of independent manipulations of D1- and D2-positive cells, I hypothesized that *Akt* would be dysregulated within D2-postive cells, which have been associated with susceptibility to stress (Francis et al., 2015; Krishnan et al., 2008). What I found, however, was that HFD exposure induced a significant reduction in *Akt* in both D1- and D2-postive cells. This effect was surprising given that it contradicts the bulk tissue gene expression data presented in Figure 21*A*, in which I reported a compounded increase in *Akt* after PS+HFD. Because *Akt* is found predominantly in microglia (Zhang et al., 2014), a major regulator of neuroinflammation after HFD exposure (Valdearcos et al., 2014), I then sorted out microglia from adolescent mice exposed to simultaneous CSDS+HFD. The qPCR revealed that *Akt* was increased as a factor of stress, following a similar pattern as the bulk tissue. These data indicate that the alterations in *Akt* may be an effect predominantly mediated by microglial cells. Lastly, I aimed to

characterize the functional role of *Akt* in mice exposed to PS+HFD simultaneously. Studies have shown that viral downregulation of *Akt* can induce resilient phenotypes (Krishnan et al., 2008; Parise et al., 2021). I utilized a short hairpin for *Akt* (sh*Akt*), designed to reduce its mRNA levels. As expected, all the stress-exposed mice in the scrambled control virus group demonstrated normal avoidant phenotypes after CSDS (see Figure 25*B*). Additionally, in the sh*Akt* group, the PS+CD-exposed mice showed no social avoidance thus indicative of a resilient phenotype. Interestingly, I found that the PS+HFD-exposed mice did not adopt the resilient phenotype and were significantly avoidant. These data indicate that HFD may be inducing and maintaining stress susceptibility via the *Akt* pathway within the NAc. Although speculative, this could also account for why FLX is not as efficacious in PS+HFD-exposed mice from Chapter 3, Figure 13*B*.

My studies do have some limitations. One of the major limitations of this dissertation is the apparent lack of power. This may be intrinsic to the paradigms utilized in this series of studies. Given the amount of variation induced by both stress and diet, even after using subject numbers that would have given more reliable results using other paradigms, increasing the power of these experimental studies should help delineate susceptible vs. resilient as well as obesogenic vs. non-obesogenic mice. Additionally, more relevant correlations between neurobiological markers, circulating factors, and behavioral outputs could be made and would be of great value to the field of nutritional neuroscience. Another limitation is the lack of female subjects, as it has been shown that females not only suffer from this comorbidity but may have a worse prognosis compared to their male counterparts (Lin et al., 2014; Mannan et al., 2016). It is important that other researchers not only replicate these findings, but also expand upon the short and long-term metabolic abnormalities and correlate them to various behavioral outputs (e.g., depression-like, anxiety-like,

cognitive, etc.) between males and females. This issue is not a monolith, as it requires a diverse range of expertise to address the multi-system dysregulation that occurs after experiences with stress or poor diet. Given this, further characterization from many avenues of research is needed to create better potential therapeutics for either MDD, obesity or the co-occurrence of both. The lack of definitive mechanism found was not unexpected given the lack of research on the topic and the complexity of the issue.

In conclusion, I have demonstrated that various aspects of the metabolic-mood syndrome can be examined using adolescent models of concurrent stress and western-style HFDs. I found that exposure to early-life stress and HFD lead to compounding deficits in weight gain leading to obesogenic-like phenotypes. These deficits were accompanied by alterations in reward sensitivity, *Akt* signaling, inflammatory markers, and a decreased behavioral responsivity to the antidepressant effects of FLX. I postulate that both diets high in fat and/or carbohydrates and chronic stress can induce intracellular signaling changes within limbic regions of the brain and alter microbiota composition leading to both physiological and mood deficits. These alterations within the brain and the gut are likely mediated at least in part, through the immune system, which can be dysregulated by both diet and stress and when combined, the compounded inflammatory response may lead to other secondary conditions that can precipitate obesity during depression, or vice-versa. The involvement of non-neuronal subtypes is key to unravelling this issue as microglia and astrocytes maintain the barrier and protect the environment of the brain. Ultimately, the goal of my work was to establish a new avenue of investigation that will allow for the further categorization of the comorbidity between diet- and stress-related disorders. Through education, proper prevention, and self-care during the sensitive period of adolescence can prevent further

comorbidities, discovering and developing new therapeutics that target the common biological factors could improve quality of life for millions of people around the world suffering from the compounding effects of the metabolic-mood syndrome.

Table 1: RT-qPCR primer sequences.

Gene	Primer sequence (Forward)	Primer sequence (Reverse)
Gapdh	5′-AGGTCGGTGTGAACGGATTTG-3′	5′-TGTAGACCATGTAGTTGAGGTCA-3′
Akt	5′-ATCCCCTCAACAACTTCTCAGT-3′	5′-CTTCCGTCCACTCTTCTCTTTC-3′
Erk1	5′-GTACATCGGAGAAGGCGCCTAC-3′	5′-TTGTAAAGGTCCGTCTCCAT-3′
Erk2	5′-TTGCTTTCTCTCCCGCACAAA-3′	5′-AGAGCCTGTTCAACTTCAATCC-3′
***Gsk3*B**	5′-TGGCAGCAAGGTAACCACAG-3′	5′-CGGTTCTTAAATCGCTTGTCCTG-3′
Ccl2	5′-AGGTCCCATGTCATGCTTCTGG-3′	5′-CTGCTGCTGGTGATCCTCTTG-3′
NfκB	5′-GCTGCCAAAGAAGGACACGACA-3′	5′-GGCAGGCTATTGCTCATCACAG-3′

Table 2: Diet compositions.

Diet	Abbreviation	Source	Energy Density	Calories from Fat	Calories from Carbohydrate	Calories from Protein
Normal Chow	NC	Teklad Rodent Diet; 8604	3.0 kcal/g	14% kcal	54% kcal from starches	32% kcal
Control Diet	CD	Research Diets; D12450K;	3.82 kcal/g	10% kcal	70% kcal from starches	20% kcal
Low-fat / high - carbohydrate diet	LFD	Research Diets; D12450H;	3.82 kcal/g	10% kcal from lard	70% kcal from sucrose	20% kcal
High-fat / high - carbohydrate diet	HFD	Research Diets; D12451	4.72 kcal/g	45% kcal from lard	35% kcal from sucrose	20% kcal

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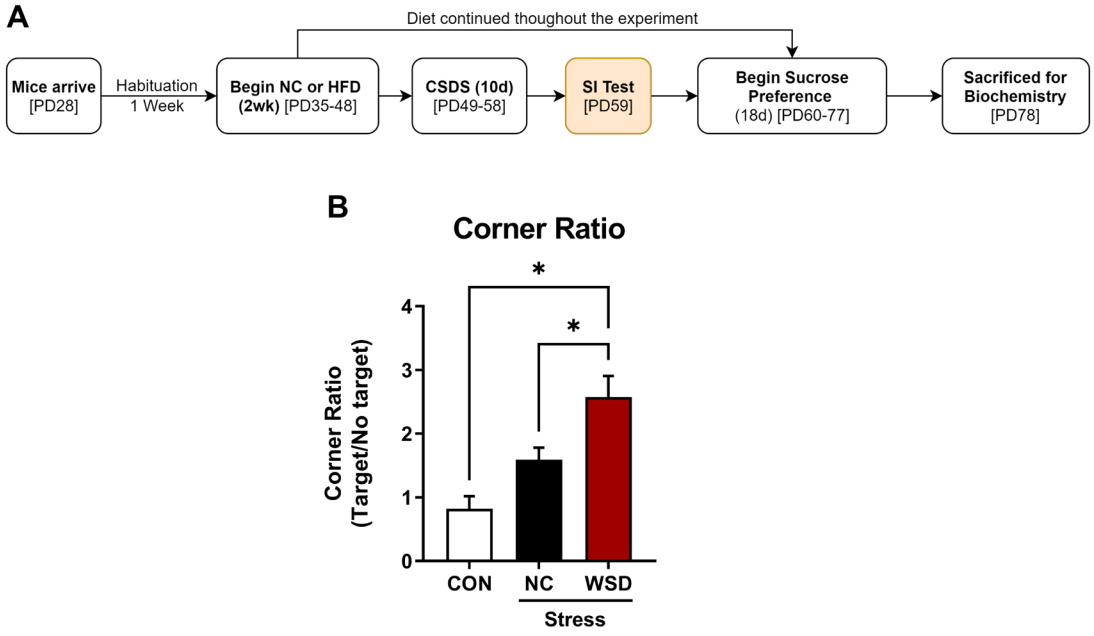
APPENDIX A



Exposure to 2 weeks of HFD before CSDS leads to increases in time spent in corners.

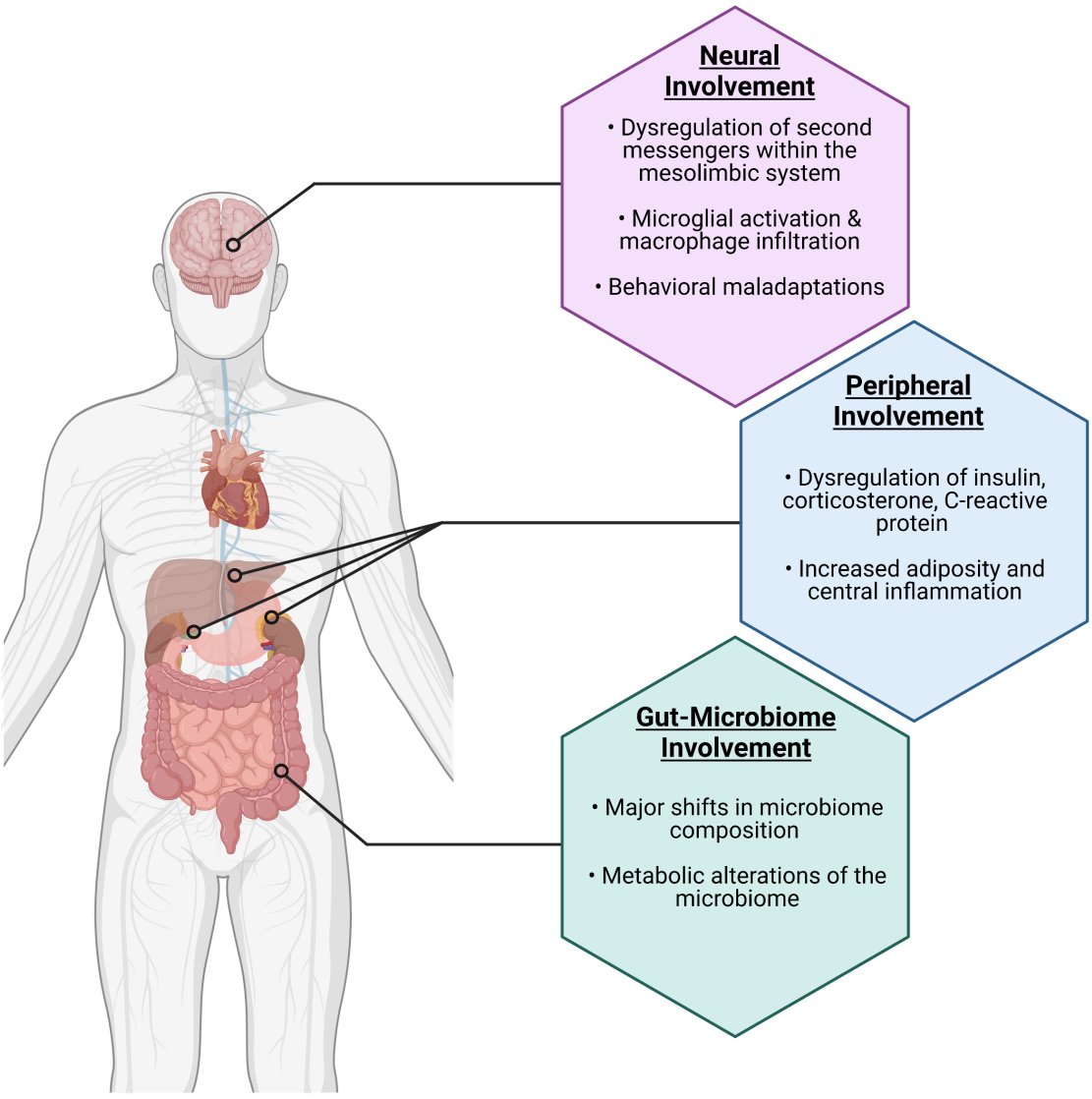
Experimental design. After the habituation period, the mice were moved into single housing (PD35) with NC or HFD (n=12/group) available ad libitum for 8 weeks (PD35-77). By the end of the first 2 weeks of diet exposure, the mice were exposed to CSDS (PS) for 10 days, followed by the SIT 24 hours after the last defeat exposure (Appendix. 1*A*).

Social interaction. The impact of NC or HFD before CSDS on social interaction was examined. A parallel second group of mice (n=5), no-diet/no-stress, was used as controls (CON) for the SIT (Appendix. 1*B*). One-way ANOVA showed significant differences in time spent in corners as a function of stress ($F_{(2,46)}$=6.151; p<0.0043), indicating that the NC- and HFD-exposed mice avoided the social target and spent more

time in corners when target was present (a depression-like phenotype). Interestingly, HFD-exposed mice spent more time in corners than NC-exposed stressed mice as differences in corner ratio between the diet groups were detected ($p<0.05$).

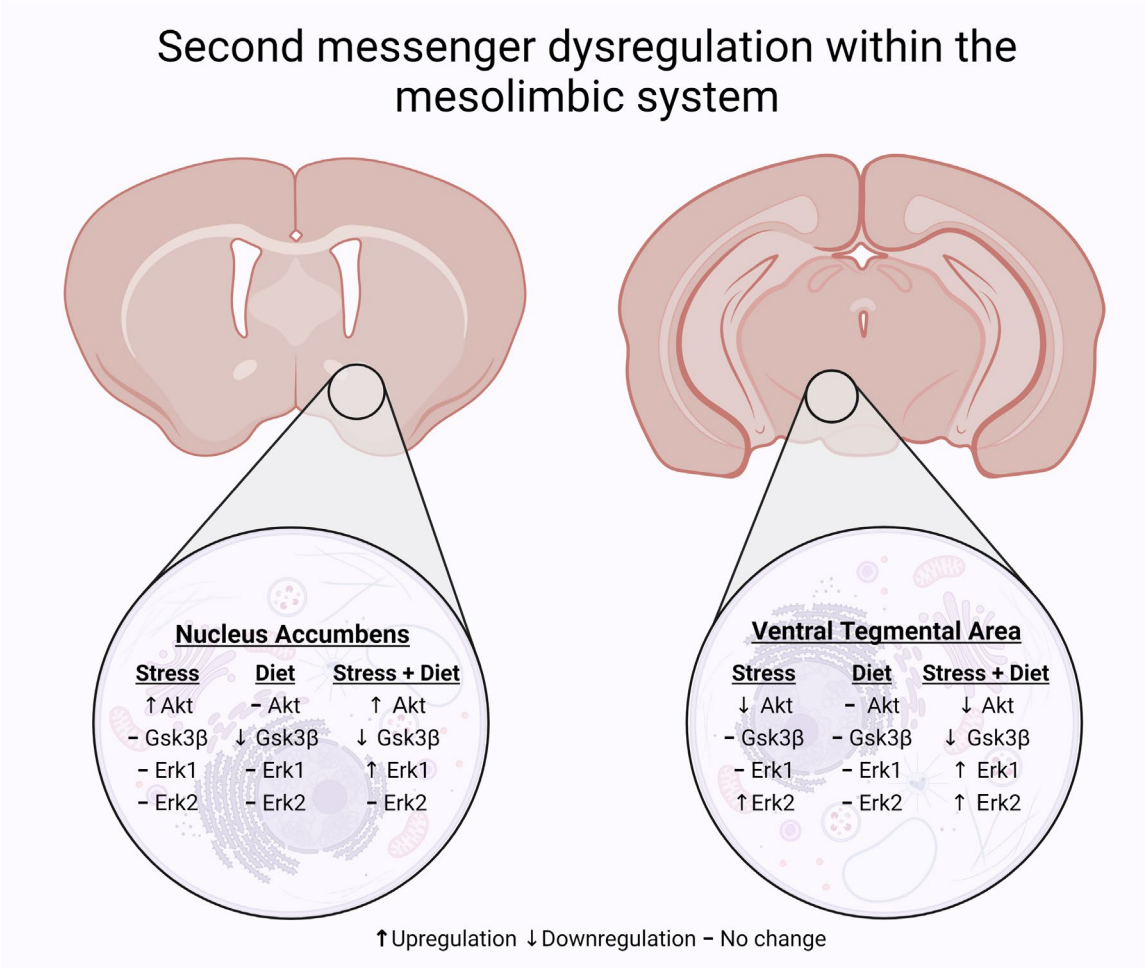
APPENDIX B

Appendix B1: Simplified overview of Metabolic mood syndrome.



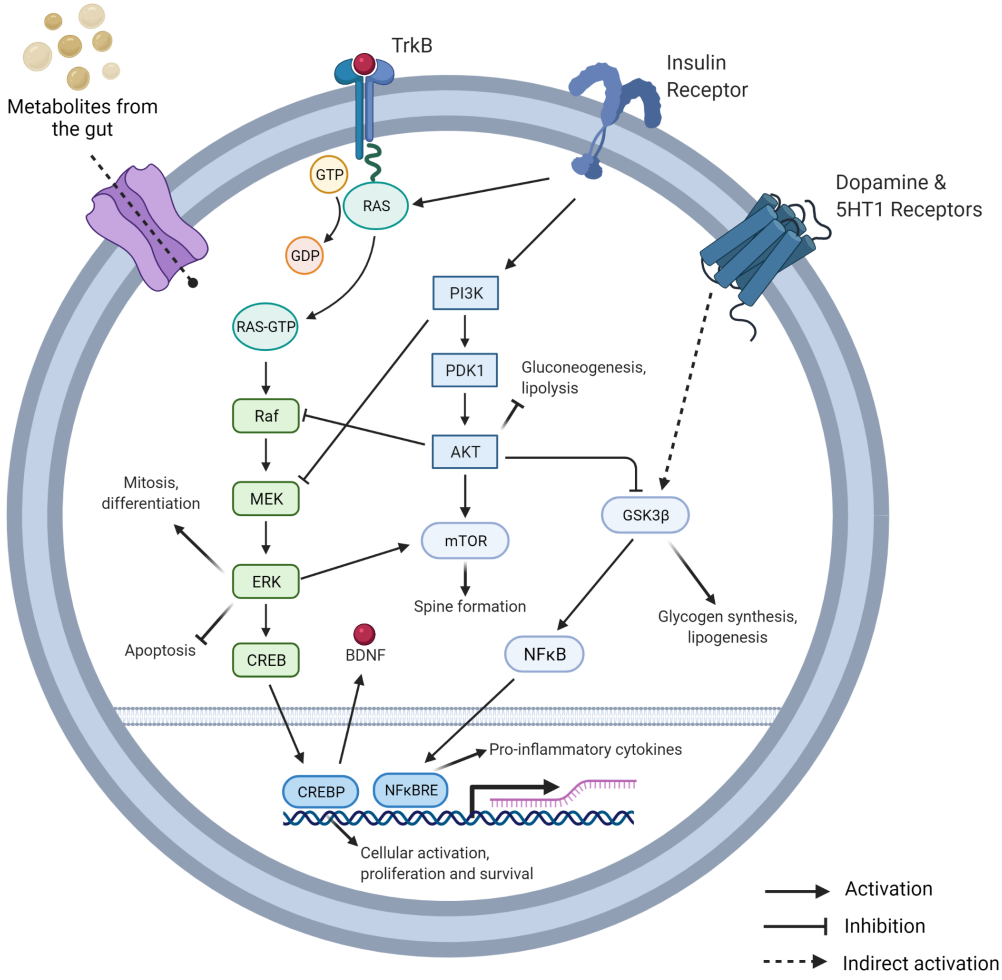
The comorbidity between depression and obesity is considered to be part of a phenomenon known as Metabolic Mood Syndrome involving multiple organ systems such as the brain, liver, pancreas, adrenal, and gastrointestinal tract. Interplay between these systems in consequence to chronic stress and western-style diets contribute to physiological and behavioral maladaptations.

Appendix B2: Overview of second messenger system dysregulation within the mesolimbic system after CSDS and HFD-exposure during adolesence.



Adolescent mice were exposed to chronic social defeat stress (CSDS) and high-fat and high-carbohydrate (HFD) and gene expression for *Akt, Gsk3β, Erk1/2*.was measured within mesolimbic regions of the brain (e.g., Nucleus Accumbens [NAc] and Ventral Tegmental Area [VTA]. Differential regulation of these genes in response to stress, diet, and the combination of both is shown above.

Appendix B3: Simplified representation of canonical intracellular signaling pathways within the brain.



Kinases such as protein kinase B (AKT), extracellular signal-regulated kinase (*ERK1/2*), and glycogen synthase kinase (GSK3β) are critical mediators of cellular response and adaptation to external stimuli. Downstream effectors such as CREB, mTOR and NFκB

have broad ranging implications that maintain homeostasis. The graphic above illustrates how insults such as chronic stress and western style diet can have extending effects given the interconnectedness of these intracellular targets and their various metabolic responsibilities.