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ENERGETIC STRESS:
THE RECIPROCAL RELATIONSHIP BETWEEN ENERGY AVAILABILITY AND THE STRESS RESPONSE

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B.A., Pomona College, 2005

Advisor: Gretchen N. Neigh, PhD

An abstract of a dissertation submitted to the Faculty of the

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ABSTRACT

ENERGETIC STRESS:
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By

Constance Scott Harrell Shreckengost

Cerebral structure and function are intrinsically dependent on the brain's ability to avail itself of energy. In turn, perturbations to energy availability alter cerebral structure and function. The stress response, mediated through the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system, shifts energy allocation to maximize availability to structures of most need. Under chronic stress, these shifts in energy availability can become maladaptive. HPA axis dysfunction related to chronic stress is linked to disorders including obesity, diabetes, and cardiovascular disease. As these epidemics of obesity and metabolic syndrome continue to expand, investigating the reciprocal relationship between energy availability and the stress response is imperative to addressing the neuropsychiatric implications of these conditions. In Part One of this dissertation, I examine the environmental and hormonal influences on markers of cerebral metabolism, namely, glucose transporters. I demonstrate that psychosocial and inflammatory stressors modulate cerebral glucose transporter expression in a region- and sex-dependent manner. Ovarian steroids regulate regional expression of these same transporters, which may be relevant to sex differences in the stress response. In Part Two, after reviewing the diverse contributors to cerebral metabolism, I shift focus to examine how a dietary influence on energy availability – i.e., a high-fructose diet – alters the stress response. I present evidence of the effects of this diet on metabolic, hormonal, and behavioral outcomes, indicating that high-fructose diet initiated during adolescence promotes a negative metabolic phenotype as well as depressive- and anxiety-like behavior. Periadolescent high-fructose diet also remodels the hypothalamic transcriptome, with a particular impact on POMC processing and additional effects on inflammatory and dopaminergic pathways. In addition, high-fructose diet remodels cerebral vasculature without affecting behavioral outcomes after cerebral ischemia. Taken together, the evidence presented indicates that exposure to stressors including psychosocial stress, inflammatory stress, and physical injury shift brain and behavior to alter energy availability. On the other hand, changes in energy availability, elicited through a high-fructose diet, can alter the stress response. Thus, this dissertation demonstrates that the reciprocal relationship between energy availability and the stress response is far-reaching, affecting the immune system, the endocrine system, limbic circuitry, and cerebral vasculature.

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If someone had asked me when I graduated college in 2005 what I would be doing ten years from then (as I'm sure someone did), I would never have guessed that I would be submitting a dissertation as partial fulfillment of the requirements for the degree of PhD in Neuroscience. Yet here I am, in 2015, and looking back I can't believe that I made it onto this path. I could not have done it without the encouragement and support of so many people around me. First, I would like to thank my family, especially my parents, Evans Harrell and Charity Scott, who encouraged me to take on this endeavor even when it was just a crazy inkling of an idea. I also owe a huge debt of gratitude to Richard Nichols and Shawn Hochman, for being brave enough to take me on when I really had no lab experience, little coursework, and no clue what I was doing. Without your leap of faith, I wouldn't be here. Keith Tansey deserves many thanks as well, as he gave me the skills I needed to learn how to set up an experiment and begin to think for myself.

I am forever indebted to my advisor, Gretchen N. Neigh, for taking me on as a student and helping me to learn when to run forward full-throttle and when running forward might mean running off a cliff. I would additionally like to thank all of my committee members for insight support; Mark Wilson, for your wisdom as my project evolved; David Weinshenker, for pushing me further whether running or in lab; Malù Tansey, for your help in all aspects from theoretical to technical; and Kerry Ressler, for your willing ear and direct advice. I'd like to thank my colleagues in the lab, as none of the work in this dissertation would have been possible without them, in particular our lab manager, Sean Kelly. Moreover, the undergraduates with whom I have had the pleasure of working have made my experience in the lab as wonderful as it as been. They not only made this dissertation possible, but also made me a better scientist and person along the way. Jillybeth Burgado deserves a special shout-out as she was a part of my experience all four years. Of course, I can't forget the rats that gave their lives for this work, and I hope that they have not died in vain. Finally, I'd like thank my husband, Jacob Shreckengost, whom I met in the lab and who has supported me this whole way through, through both tears and joy.

TABLE OF CONTENTS

GENERAL INTRODUCTION…………………………………………………………..…………………...1

1. Energetic stress: Disruptions to energy homeostasis alter the stress response…………….……..2
   - ***1.0*** Abstract - 2
   - ***1.1*** Introduction - 3
   - ***1.2*** Overview of the hypothalamic role in regulating energy and the stress response - 4
     - ***1.2.1*** The hypothalamus as a regulator of energy homeostasis – 5
     - ***1.2.2*** Relationship between metabolism and stress-induced activation of the HPA axis -6
   - ***1.3*** Influence of energy homeostasis on the stress response -7
     - ***1.3.1*** Dietary imbalance disrupts HPA axis signaling - 8
     - ***1.3.2*** Disrupted energy homeostasis impairs central glucocorticoid signaling - 10
     - ***1.3.3*** Disruption of energy homeostasis alters glucocorticoid signaling in peripheral tissues – 12
     - ***1.3.4*** Disruptions to energy homeostasis induce hyperactivity of the sympathetic nervous system - 13
     - ***1.3.5*** Change in energy homeostasis disrupts signaling of neuroendocrine and neuropeptide factors that interact with the HPA axis – 15
     - ***1.3.6*** Disruptions to energy homeostasis alter the HPA axis response to inflammatory stimuli – 17
     - ***1.3.7*** Disruptions of energy homeostasis modulate the gut-brain axis to dysregulated the HPA axis – 19
   - ***1.4*** Discussion – 23

PART ONE…………………………………………………………………………………….27

2. Psychosocial and Inflammatory Stressors Modulate Regional Glucose Transporter Expression in a Sexually-Dimorphic Manner……………………………………………………………..35
   - ***2.1*** Abstract - 35
   - ***2.1*** Introduction - 36
   - ***2.2*** Materials and Methods – 39
     - ***2.2.1*** Animals - 39
     - ***2.2.2*** Experimental Design – 40
     - ***2.2.3*** Mixed Modality Chronic Stress – 41
     - ***2.2.4*** Vaginal Lavage – 41
     - ***2.2.5*** Quantitative RT-PCR – 42
     - ***2.2.6*** Statistical Analysis – 43
   - ***2.3*** Results - 43
     - ***2.3.1*** Hypothalamic GLUT1 and GLUT4 mRNA abundance increases with age and is sexually dimorphic - 43
     - ***2.3.2*** Hippocampal mRNA Abundance of GLUT3, GLUT4, and GLUT5 decreases with age in males and females - 44
     - ***2.3.3*** Neither sex nor age impact mRNA abundance of GLUTs in the amygdala or prefrontal cortex - 44
     - ***2.3.4*** Chronic stress reduces weight gain - 44

2.3.5 Chronic stress interacts with stress and age to alter hypothalamic GLUT mRNA abundance – 45
2.3.6 Chronic stress interacts with stress and age to increase hippocampal GLUT mRNA abundance – 45
2.3.7 Chronic stress interacts with stress and age to increase GLUT mRNA abundance in the male amygdala - 46
2.3.8 Chronic stress does not alter GLUT mRNA abundance in the prefrontal cortex - 46
2.3.9 Effects of adolescent chronic stress on hippocampal GLUT mRNA abundance persist into adulthood in a sexually dimorphic manner – 47
2.3.10 Adolescent chronic stress blunts the male increase in hippocampal GLUT mRNA abundance after LPS – 47
2.4 Discussion - 48

3. Estrous cycle and ovariectomy influence regional expression of cerebral glucose transporter isoforms…63
3.0 Abstract – 63
3.1 Introduction – 63
3.2 Materials and Methods – 66
3.2.1 Animal Husbandry – 66
3.2.2 Cycle Staging – 66
3.2.3 Plasma Hormone Analysis – 67
3.2.4 Ovariectomy – 67
3.2.5 Quantitative RT-PCR - 68
3.2.6 Statistical Analysis – 69
3.3 Results - 69
3.3.1 Estrous cycle stage was established from vaginal lavage and confirmed with uterine weights and hormone concentrations - 69
3.3.2 The hippocampus and hypothalamus demonstrate a greater relative abundance of GLUT mRNA than the prefrontal cortex - 70
3.3.3 The hippocampus and prefrontal cortex demonstrate greater cycle-dependent variation in gene expression for GLUTs than the hypothalamus – 71
3.3.4 Expression of GLUT1, 3, and 8 in the hippocampus is directly influenced by ovarian hormones – 71
3.4 Discussion – 71

INTERLUDE……………………………………………………………………………..84

4. Contributors to cerebral energy metabolism…………………………………………………85
4.0 Abstract – 85
4.1 Review

PART TWO…………………………………………………………………………………78

5. High-fructose diet initiated during adolescent development influences metabolism, behavior, and the HPA axis in male rats………………………………………………………………………92
5.0 Abstract – 92
5.1 Introduction – 93
5.2 Materials and Methods – 94

5.2.1 Animal Husbandry – 94
5.2.2 Diet and Metabolic Measurement – 95
5.2.3 Fat Pad Collection – 97
5.2.4 Mixed Modality Stress – 97
5.2.5 Glucose Tolerance Test – 98
5.2.6 Insulin Analysis - 99
5.2.7 Behavioral Testing – 99
5.2.8 Corticosterone Analysis – 100
5.2.9 Whole Transcriptome RNA sequencing – 100
5.2.10 Pathway Analysis – 101
5.2.11 Quantitative RT-PCR - 102
5.2.12 Statistical Analysis – 104

5.3 Results - 104
5.3.1 Periadolescent high-fructose diet increases caloric efficiency, fasting glucose, and visceral fat pad mass - 104
5.3.2 Periadolescent high-fructose diet and stress alter weight gain and fasting glucose – 105
5.3.3 Periadolescent high-fructose diet alters gene expression of hippocampal glucose transporter-1 after a glucose challenge or acute stress – 106
5.3.4 Periadolescent high-fructose feeding does not alter gene expression of glucocorticoid signaling factors – 107
5.3.5 Periadolescent high-fructose diet increases anxiety-like behaviors -107
5.3.6 Periadolescent high-fructose diet increases depressive-like behaviors - 108
5.3.7 Periadolescent high-fructose diet interacts with acute stress to alter plasma corticosterone - 109
5.3.8 Periadolescent high-fructose diet remodels the hypothalamic transcriptome with greatest impact on POMC processing – 109
5.3.9 High-fructose diet upregulates hypothalamic expression of *Pomc* and *Crfr1* when consumed during periadolescence but only *Pomc* when consumed during adulthood - 110

5.4 Discussion – 111

6. High-fructose diet initiated during adolescent development increases expression of innate immune factors in male rats ……………………………………………………………………140

6.0 Abstract - 140
6.1 Introduction – 141
6.2 Materials and Methods – 143
6.2.1 Animal Husbandry – 143
6.2.2 Diet – 144
6.2.3 Whole Transcriptome RNA-sequencing – 144
6.2.4 Pathway Analysis – 145
6.2.5 Quantitative RT-PCR - 146
6.2.6 Immunoblotting – 147
6.2.7 Statistical Analysis – 148

6.3 Results - 148
6.3.1 High-fructose diet consumption remodels hypothalamic gene expression – 149
6.3.2 Hypothalamic immune response and inflammatory pathways are significantly enriched with transcripts altered by fructose consumption - 149

6.3.3 Hypothalamic expression of classical, lectin-induced, and alternative complement pathways are significantly enriched with transcripts altered by fructose consumption - 149
6.3.4 Rats fed a high-fructose diet during adolescent development have increased expression of complement components *C4b* and *Cfb* - 149
6.3.5 Rats fed a high-fructose diet only in adulthood do not show increased hypothalamic expression of complement components *C4b* and *Cfb* - 150
6.3.6 Increased expression of complement components *C4b* and *Cfb* extends from the hypothalamus to the hippocampus in rats fed a high-fructose diet during periadolescence but not adulthood – 150
6.3.7 Synaptophysin and post-synaptic density 95 are not differentially expressed in the hippocampus of periadolescent fructose-fed rats – 151
6.3.8 Expression of complement factors predicts expression of synaptic markers in the hippocampus – 151
6.4 Discussion – 152

7. High-fructose diet initiated during adolescent development alters hypothalamic expression of mRNA related to dopamine synthesis and signaling without affecting protein expression in male rats……………………………………………………………………………………….172
7.0 Abstract – 172
7.1 Materials and Methods - 175
7.1.1 Animal Husbandry – 176
7.1.2 Diet – 176
7.1.3 Whole-transcriptome RNA sequencing – 176
7.1.4 Pathway analysis – 177
7.1.5 Prolactin Analysis - 178
7.1.6 Immunoblotting – 178
7.1.7 Statistical Analysis – 179
7.2 Results – 180
7.2.1 Fructose and chow diets determine clustering of the hypothalamic transcriptome - 180
7.2.2 Periadolescent fructose-feeding alters expression of genes in dopamine signaling pathways - 180
7.2.3 Plasma prolactin is not significantly altered in either periadolescent or adult fructose-fed rats - 181
7.2.4 Plasma prolactin predicts dopamine transporter, tyrosine hydroxylase, and vesicular monoamine transporter-2 gene expression in RNA-sequencing - 181
7.2.5 Tyrosine hydroxylase, vesicular monoamine transporter-2, and dopamine transporter expression were unaffected by fructose-feeding in the dorsal striatum of both periadolescent and adult rats – 182
7.2.6 Periadolescent fructose-feeding did not alter tyrosine hydroxylase or vesicular monoamine transporter-2 protein expression in the hypothalamus, nucleus accumbens, and ventral tegmental area - 182
7.3 Discussion – 183

8. High-fructose diet initiated during adolescent development promotes cerebrovascular remodeling but does not alter behavioral outcomes after middle cerebral artery occlusion in male rats ……………………199
8.0 Abstract - 199
8.1 Introduction – 199

8.2 Materials and Methods – 202
8.2.1 Animal Husbandry – 202
8.2.2 Diet – 202
8.2.3 Metabolic Assessments – 203
8.2.4 Corticosterone analysis – 204
8.2.5 Quantitative RT-PCR – 204
8.2.6 Blood-brain-barrier Permeability – 205
8.2.7 Vascular Length - 206
8.2.8 Surgery – 207
8.2.9 Neurologic Behavior Assessment – 207
8.2.10 Affective Behavior Assessment – 209
8.2.11 Lesion Volume Assessment – 210
8.2.12 Statistical Analysis – 211
8.3 Results – 211
8.3.1 Fructose alters basal metabolism and decreases weight loss after surgery - 211
8.3.2 Fructose increases hippocampal gene expression of *Vegfa* – 212
8.3.3 Fructose increases vascular density in CA1 of the hippocampus – 212
8.3.4 Fructose does not alter blood-brain-barrier permeability - 212
8.3.5 Fructose and acute stress increase corticosterone irrespective of surgery – 212
8.3.6 Fructose does not exacerbate MCAO-induced impairments in neurologic behavior – 213
8.3.7 Fructose promotes anxiety-like behavior in both sham and MCAO-affected animals - 214
8.3.8 Greater lesion volume correlates with an active coping strategy in the forced swim test - 215
8.3.9 Fructose does not change lesion volume after MCAO - 184
8.4 Discussion – 215

GENERAL DISCUSSION……………………………………………………………………..234

9. Energetic Stress: Expensive effects on body and brain………………………………………235
9.0 Abstract - 235
9.1 Introduction - 235
9.2 Stress effects on a marker of cerebral energy metabolism – 238
9.2.1 Psychosocial and inflammatory stress change expression of cerebral glucose transporters in a region-, age-, and sex- specific manner - 238
9.2.2 Ovarian hormones modulate expression of cerebral glucose transporters in a region- and isoform- specific manner - 240
9.2.3 Future directions in stress effects on GLUT – 241
9.3 Effects of shifting energy homeostasis on the stress response – 242
9.3.1 Fructose as an energetic stressor – 242
9.3.2 Metabolic & behavioral effects of a high-fructose diet – 245
9.3.3 Effects of fructose on the hypothalamic transcriptome – 246
9.3.4 Energy homeostasis and HPA axis effects on outcomes after ischemic injury – 249
9.3.5 Future directions in the study of the effects of a high-fructose diet on the stress response - 250
9.4 Conclusion – 250

REFERENCES……………………………………………………………………….254

LIST OF FIGURES

Chapter One

1.1 Disruptions to Energy homeostasis Alter the Stress Response through Multiple Mechanisms - 29

Chapter Two

2.1 Adolescent male rats express less GLUT1 and GLUT4 in the hypothalamus than either adult males or females - 56
2.2 Age determines gene expression of GLUT3, GLUT4, and GLUT8 in the hippocampus, regardless of sex - 57
2.3 Stress differentially alters GLUT expression in the hypothalamus of male and female rats - 58
2.4 Chronic stress, age, and sex interact to dictate GLUT expression in the hippocampus-59
2.5 Amygdalar expression of GLUTs is largely impervious to sex, age, and stress -60
2.6 Effects of adolescent chronic stress on hippocampal GLUT mRNA abundance persist into adulthood in a sexually dimorphic manner - 61
2.7 Adolescent chronic stress blunts the male increase in hippocampal GLUT mRNA abundance after LPS - 62

Chapter Three

3.1 Uterine weight and P:E ratio fluctuate across estrous cycle stages - 78
3.2 The hippocampus and hypothalamus demonstrate a greater relative abundance of GLUT mRNA than the prefrontal cortex - 79
3.3 GLUT1 mRNA abundance is greatest and GLUT4 mRNA abundance is lowest in all regions analyzed - 80
3.4 The hippocampus and prefrontal cortex demonstrate greater cycle-dependent variation in gene expression for GLUT1 than the hypothalamus - 81
3.5 Hippocampal mRNA abundance of GLUT3 and GLUT4 varies by cycle stage and peaks in pro-estrus and estrus respectively - 82
3.6 Expression of GLUT1, 3, and 8 in the hippocampus is directly influences by ovarian hormones – 83

Chapter Five

5.1 Periadolescent high-fructose diet alters metabolic parameters – 122
5.2 Adolescent stress and periadolescent fructose affect weight gain and blood glucose – 123
5.3 Periadolescent high-fructose diet alters gene expression of hippocampal glucose transporter-1 after a glucose challenge or acute stress – 124
5.4 Periadolescent high-fructose diet increases anxiety-like behavior – 125
5.5 Periadolescent high-fructose diet increases depressive-like behavior – 126
5.6 Periadolescent high-fructose diet elevates baseline corticosterone – 127
5.7 Periadolescent high-fructose diet remodels the hypothalamic transcriptome – 128
5.8 Periadolescent high-fructose diet remodels CRF signaling and POMC processing – 129

5.9 High-fructose diet upregulates hypothalamic expression of *Pomc* and *Crfr1* when consumed during periadolescence but only *Pomc* when consumed during adulthood - 130
Supplemental 5.1 Experimental Timelines - 131

Chapter Six

6.1 Periadolescent high-fructose diet remodels the hypothalamic transcriptome – 164
6.2 Periadolescent high-fructose diet increases expression of gene transcripts in all three complement pathways – 165
6.3 Periadolescent but not adult consumption of a high-fructose diet elevates hypothalamic expression of complement components *C4b* and *Cfb* – 166
6.4 Periadolescent but not adult consumption of a high-fructose diet elevates hippocampal expression of complement components *C4b* and *Cfb* – 167
6.5 Hypothalamic C4b and hippocampal Cfb are elevated without the contribution of blood factors – 168
6.6 Synaptophysin and post-synaptic density 95 are not differentially expressed in the hippocampus of periadolescent fructose-fed rats - 169

Chapter Seven

7.1 Principal component analysis demonstrates separation of hypothalamic gene expression based on diet type - 190
7.2 Plasma prolactin is not significantly altered in either periadolescent or adult fructose-fed rats – 191
7.3 Tyrosine hydroxylase, vesicular monoamine transporter-2, and dopamine transporter protein expression were unaffected by fructose-feeding in the dorsal striatum of both periadolescent and adult rats – 192
7.4 Periadolescent fructose feeding did not alter tyrosine hydroxylase or vesicular monoamine transporter-2 protein expression in the hypothalamus, nucleus accumbens, and ventral tegmental area – 193

Chapter Eight

8.1 Dietary, surgical, and behavioral timelines for cohorts used in this study – 223
8.2 Fructose alters basal metabolism and decreases weight loss after surgery – 224
8.3 Fructose promotes hippocampal vascularization – 225
8.4 Fructose does not alter amygdalar vascularization – 226
8.5 Fructose does not alter blood-brain-barrier permeability – 227
8.6 Fructose and acute stress increase corticosterone irrespective of surgery – 228
8.7 Fructose does not exacerbate MCAO-induced impairments in neurologic behavior - 229
8.8 Baseline parameters in neurologic behavior – 230
8.9 Fructose promotes anxiety-like behavior in both sham and MCAO-affected animals- 231
8.10 Greater lesion volume correlates with an active coping strategy in the forced swim test – 232
8.11 Fructose does not alter lesion volume after MCAO – 233

Chapter Nine

9.1 Reciprocal relationship between energy homeostasis and the stress response - 253

LIST OF TABLES

Chapter One

1.1 Hypothalamic Nuclei – 30
1.2 Neuroendocrine and Neuropeptide Regulators of Energy Homeostasis – 31
1.3 Effects of Energetic Stress of the HPA Axis and the Stress Response - 32

Chapter Two

2.1 Summary of cerebral glucose transporters potentially involved in neurological disease or stress responses – 55

Chapter Three

3.1 Cerebral glucose transporter isoforms analyzed with respect to ovarian hormones – 77

Chapter Five

Supplemental 5.1 Cohort distribution and use – 132
Supplemental 5.2 Dietary composition – 133
Supplemental 5.3 Pathways significantly enriched by differentially expressed genes between periadolescent chow and fructose-fed cohorts – 134
Supplemental 5.4 Networks significantly enriched by differentially expressed genes between the periadolescent chow and fructose-fed cohorts - 135
Supplemental 5.1 Mean weekly weights in metabolic periadolescent and adult cohorts – 136
Supplemental 5.2 Mean weekly kCal consumed in periadolescent and adult cohorts – 137
Supplemental 5.3 Mean weekly fasting blood glucose in periadolescent and adult cohorts – 139

Chapter Six

6.1 Cohort distribution and use – 160
6.2 Immune and inflammatory pathway map folders rank highly in analysis of diet-dependent differentially expressed genes - 161
6.3 Significantly enriched pathways by diet-dependent differentially expressed genes – 162
6.4 Hippocampal *C1qa* and *Cfb* predict synaptophysin expression - 170
6.5 Hippocampal *C4b* and *Cfb* predict PSD95 expression – 171

Chapter Seven

7.1 Cohort distribution and use – 189
7.2 Loading factors from Principal Component Analysis of Hypothalamic RNA sequencing in Chow-Fed and Fructose-Fed Rats – 194
7.3 Dopamine Synthesis and Signaling Related Genes: Relative Hypothalamic Expression between Chow- and Fructose-Fed Animals – 195
7.4 Plasma prolactin predicts dopamine transporter expression in RNA-sequencing – 196
7.5 Plasma prolactin predicts tyrosine hydroxylase expression in RNA-sequencing – 197

7.6 Plasma prolactin predicts vesicular monoamine transporter-2 expression in RNA-sequencing – 198

Chapter Eight

8.1 Cohort distribution and use - 222

INTRODUCTION

Chapter One:

Energetic stress:

Disruptions to energy availability alter the stress response

Adapted from:

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Disruptions to energy availability alter the stress response.

In preparation.

1.0 Abstract

The worldwide epidemic of metabolic disorders and the recognized burden of mental health have driven increased research into the relationship between the two. A maladaptive stress response is implicated in both mental health disorders and metabolic disorders, implicating the hypothalamic-pituitary-adrenal (HPA) axis as a key mediator of this relationship. This review explores how an altered energetic state, such as obesity or diabetes, affects the stress response and the HPA axis in particular. We propose that changes in energetic state or energetic demands can result in “energetic stress” that can, if prolonged, lead to a dysfunctional stress response. In this review, overview of the role of the hypothalamus in modulating energy homeostasis, and then briefly discuss the relationship between metabolism and stress-induced activation of the HPA axis. Next, we examine seven mechanisms whereby energetic stress interacts with neuroendocrine stress response systems, including by altering both the central and peripheral HPA axis; by nutrient-induced changes in glucocorticoid signaling; by impacting the sympathetic nervous system; through changes in other neuroendocrine factors; by inducing inflammatory changes; and by altering the gut-brain axis. Recognizing these effects of energetic stress can drive novel therapies and prevention strategies for mental health disorders, including dietary

intervention, probiotics, and even fecal transplant. However, greater research is needed to clarify the roles that sex and sex hormones as well as developmental status play in modulating the effects of energetic stress.

1.1. Introduction

"If we break up a living organism by isolating its different parts it is only for the sake of ease in analysis and by no means in order to consider them separately. Indeed when we wish to ascribe to a physiological quality its value and true significance we must always refer it to this whole and draw our final conclusions only in relation to the effects in the whole."
– Claude Bernard, "An Introduction to the Study of Experimental Medicine," 1927 (translated in Bernard (1980))

From "gut feelings" to "heart-broken" to "butterflies in your stomach," the English language acknowledges the profound relationship between our physical and mental reactions to the external world. Though neuroscience has an historical tendency to separate its study of the nervous system and neurological disorders from the rest of the body, there is a growing awareness of the essential role that reciprocal interactions between systems may play in moderating physiology both in homeostasis and under stress. In particular, the worldwide epidemic of metabolic disorders (Ogden, Carroll, Kit, & Flegal, 2014; Swinburn et al., 2011) and the recognized burden of mental health disorders (Ustun, Ayuso-Mateos, Chatterji, Mathers, & Murray, 2004) have led to an increased interest in the potential relationship between the two. Multiple clinical studies have linked disorders such as depression and post-traumatic stress disorder to obesity and diabetes (R. J. Anderson, Freedland, Clouse, & Lustman, 2001; Weiss et al., 2011). Mechanistically, a maladaptive stress response is implicated in both mental health disorders and metabolic disorders, implicating the hypothalamic-pituitary-adrenal (HPA) axis as a

key mediator of this relationship. Recently, the International Diabetes Federation even highlighted the need for research into the role of the HPA axis in its review of the Metabolic Syndrome (IDF, 2006).

As several other reviews (Bjorntorp & Rosmond, 2000; Reagan, 2012; Rosmond, 2005) and meta-analyses (Blaine, 2008) have focused on the potential role of stress and mental health disorders to induce alterations in metabolic function, this review will instead focus on the role of metabolic dysfunction and other disruptions to energy availability to alter stress response and HPA axis. Just as disruptions to the social and physical environment can promote psychosocial or mechanical stress, changes in energetic state or energetic demands can result in “energetic stress” as the body is faced with resource-based trade-offs leading to widespread effects at the cellular and systems levels (Bland & Birnbaum, 2011; B. F. Miller & Hamilton, 2012; Thompson, Muller, Kahlenberg, & Wrangham, 2010). Thus, states of metabolic dysfunction, including obesity (excess energy storage), diabetes (altered usage and storage of energy), and irritable bowel syndrome (resulting in impaired uptake and usage of energy), all classify as energetic stressors. We will begin with an overview of the role of the hypothalamus in modulating energy homeostasis, and then briefly discuss the relationship between metabolism and stress-induced activation of the HPA axis. Next, we will discuss potential mechanisms whereby energetic alterations can moderate changes in the stress response (**Figure 1.1**), including by altering both the central and peripheral HPA axis; by nutrient-induced changes in glucocorticoid signaling; by impacting the sympathetic nervous system; through changes in other neuroendocrine factors; by inducing inflammatory changes; and by altering the gut-brain axis. Finally, we will conclude with discussion to guide future research and potential clinical therapy.

1.2 Overview of the hypothalamic role in regulating energy and the stress response

1.2.1 The hypothalamus as regulator of energy availability The responsibility for controlling energetic availability is shared by multiple peripheral organs as well as several brain regions. However, most research and interest in this field has centered on the hypothalamus. This region encompasses multiple nuclei that communicate using over 50 neurochemical molecules (Williams et al., 2001; Williams, Harrold, & Cutler, 2000), many of which are involved in the control of energy and feeding. The main neuroanatomical subregions of the hypothalamus involved in energy regulation are outlined in **Table 1.1.** Historical views of the control of food intake based on lesion models have traditionally labeled the ventromedial nucleus of the hypothalamus (VMH) as the "satiety center," while labeling the lateral hypothalamus (LH) the "hunger center" (C. B. Saper, Chou, & Elmquist, 2002). However, research in the 1990s paved the way for a more nuanced understanding of energy homeostasis and body weight regulation (C. B. Saper et al., 2002; Schwartz, Woods, Porte, Seeley, & Baskin, 2000). These advances began with identification of the gene for the adipose-derived hormone leptin and its receptor in 1994 and 1995 (Tartaglia et al., 1995) and continued as scientists gained insight into the role of ghrelin and other gut hormones as well as extended knowledge of hypothalamic peptides, such as Neuropeptide Y (NPY) and Agouti-Related Peptide (AgRP), and their receptors. A number of these key feeding-related neuroendocrine and neuropeptides are outlined in **Table 1.2.**

Though current knowledge is far from complete, we now understand the multiple drives that can result in feeding. These can include circadian drives, mediated through the suprachiasmatic nucleus (SCN); sensory drives, mediated by olfactory, gustatory, and mechanical stimuli; hedonic drives, mediated by the mesolimbic system and its connections with hypothalamic nuclei; and homeostatic drives, mediated by inputs from the periphery, including plasma leptin, insulin, and glucose levels, as well as other gut hormones and peptides (C. B. Saper et al., 2002). These drives converge at the hypothalamus, where they are synthesized to elicit various outputs, including endocrine outputs, primarily through the paraventricular nucleus

(PVN); motor outputs, through the LH; and autonomic outputs, through the LH, PVN and arcuate nucleus (ARC) (C. B. Saper et al., 2002). In a simplified model of energy homeostasis, adiposity and satiety signals, such as leptin and insulin, signal to the arcuate nucleus to inhibit anabolic NPY/AgRP pathways while stimulating catabolic proopiomelanocortin/cocaine-and-amphetamine-related-peptide (POMC/CART) pathways relayed to the PVN and LH (Schwartz et al., 2000). These signals are simultaneously integrated with signals from additional drives mentioned above. Output from the PVN and LH converges at the nucleus of the solitary tract (NTS), where information is integrated with additional ascending input from the gut. The net output results in a signal to eat or not (Schwartz et al., 2000). However, this multi-level system of inputs and outputs lends itself easily to modulation by external factors, such as stress.

1.2.2 Relationship between metabolism and stress-induced activation of the HPA axis

The HPA axis is a key effector of the stress response; however, it is worth considering the numerous additional roles played by the primary output of the HPA axis, glucocorticoids. Hans Selye named glucocorticoids for their effects on liver gluconeogenesis (Szabo, Tache, & Somogyi, 2012). Glucocorticoids play multiple important metabolic roles including regulation of gluconeogenesis, insulin sensitivity, glucose uptake, preadipocyte differentiation, and lipolysis (Carroll, Aron, Findling, & Tyrrell, 2011). Centrally, glucocorticoids, along with adrenocorticotropic hormone (ACTH), regulate the HPA axis by negative feedback via the glucocorticoid receptor (GR), transcription factors that exist on nearly every cell of the body (Panagiotakopoulos & Neigh, 2014). This negative feedback helps attenuate adrenal output in response to stressors (reviewed in (Bourke, Harrell, & Neigh, 2012; Herman & Cullinan, 1997).

In response to a stressor, the PVN of the hypothalamus releases corticotropin-releasing factor (CRF), which is transported through the hypophyseal portal system to the pars distalis of the anterior pituitary to stimulate secretion of adrenocorticotropic hormone (ACTH), a cleavage product of POMC. ACTH travels through the bloodstream to the adrenal cortex to stimulate glucocorticoid production, and in turn, glucocorticoids travel in the bloodstream bound to

corticosteroid binding globulin (CBG). The availability of glucocorticoids in target tissues depends on the activity of 11-β hydroxysteroid dehydrogenase (11βHSD), which converts inactive forms of glucocorticoids to their active forms. Active glucocorticoids mediate their effects via GR both centrally and peripherally (Schimmer & Funder, 2011). Exposure to chronic stress can alter GR expression (Chiba et al., 2012) or expression of GR co-chaperones that modulate feedback (Bourke et al., 2013) in key limbic brain regions to promote depressive- and anxiety-like behavior. When GR activity is altered or impaired, it can have important ramifications on metabolism as well, as will be discussed in the next sections.

1.3. Energetic stressors alter the HPA axis

Just as the hypothalamus and HPA axis are critical regulators of energy availability and the stress response, metabolic dysfunction, a type of energetic stressor, is associated with HPA axis impairment on multiple levels, including excessive forward drive; distorted sensitivity to negative feedback; and altered sensitivity of peripheral tissues to glucocorticoid activity (reviewed in (Seimon, Hostland, Silveira, Gibson, & Sainsbury, 2013)). These HPA axis disruptions can be modulated by changes in energy intake as well as changes in weight and fat distribution. In addition, numerous additional neuroendocrine mediators, including insulin, leptin, and several neuropeptides (C. B. Saper et al., 2002), as well as inflammatory factors, can both be affected by metabolic dysfunction and impact HPA axis activity. Collectively, this review will address seven key areas of research that explore the effects of energetic stressors on the HPA axis. These are: 1) effects of diet-based energetic stressors on the HPA axis; 2) energetic stress effects on central glucocorticoid signaling; 3) energetic stress effects on peripheral glucocorticoid signaling; 4) energetic stress effects on sympathetic nervous system hyperactivity; 5) energetic stress effects on additional neuroendocrine and neuropeptide factors; 6) energetic stress effects on

inflammation in the HPA axis; and 7) metabolic influences on the gut-brain axis. We will now turn our attention to explore these mechanisms.

1.3.1 Dietary imbalance disrupts HPA axis signaling Disruptions in energy balance may result in critical changes to an organism's HPA axis signaling, and vice versa. In healthy subjects, food consumption alone is associated with an increase in plasma cortisol (Rosmond, Holm, & Bjorntorp, 2000) and CRF-induced cortisol is strongly associated with increases in food intake (George, Khan, Briggs, & Abelson, 2010). A study of abdominally-obese vs. peripherally-obese or healthy control women demonstrated that abdominally obese women have an attenuated cortisol response to protein & lipid-dense meals but an elevated cortisol response to carbohydrate-dense meals (Vicennati, Ceroni, Gagliardi, Gambineri, & Pasquali, 2002). In contrast, peripherally obese and control women had an elevated cortisol response to the protein and lipid-dense meals but little change in response to the carbohydrate-dense meals, indicating that the HPA axis response to macronutrients may depend in part on body fat distribution.

Diet can also alter the HPA axis mediated output in response to stress, though the literature is mixed with respect to the effects of diet on HPA axis output as well as the effects of stress on feeding. In rodent models, weight loss and reduced food intake are commonly used as markers of stressor efficacy and activation of the HPA axis, but these same stressors and/or HPA axis activation can increase intake of palatable food and visceral obesity in particular (reviewed in (Adam & Epel, 2007; Dallman et al., 2003; Dallman, Pecoraro, & la Fleur, 2005)). Corticosterone replacement in adrenalectomized rats promotes sucrose consumption, as does prior exposure to sucrose, relative to unexposed unadrenalectomized rats (Bell et al., 2000). Male rats exposed to restraint stress after five to seven days of access to lard, sucrose, or both have a reduced plasma ACTH and corticosterone response as well as increased food consumption(M. T. Foster et al., 2009; Pecoraro, Reyes, Gomez, Bhargava, & Dallman, 2004), indicating the potential for energy-dense foods to dampen HPA axis output in the short term. A similar effect has been observed in humans, as two weeks of thrice daily consumption of sucrose-sweetened

beverages blunts the cortisol response to naltrexone in adult women (Tryon et al., 2015). However, six weeks of high-fat consumption in adult male mice increases plasma corticosterone in response to acute stress (S. Sharma, Fernandes, & Fulton, 2013), and eight weeks of a high-fat diet in combination with 21 days of chronic social defeat stress increases both basal and stress-induced plasma corticosterone (Balsevich et al., 2014). Female rhesus macaques given the choice of an energy dense high-fat high-sugar diet for a two-week time period also have an increased cortisol response to an acute stressor (Michopoulos, Toufexis, & Wilson, 2012). "Low quality" food intake (high consumption of meat, saturated fat, and refined sugar) is additionally associated with high basal cortisol and impaired negative feedback in the dexamethasone suppression test in humans (Duong, Cohen, & Convit, 2012). Consistent with this impaired negative feedback, eight weeks of high-fat diet exposure can reduce mRNA expression of GR and increase expression of the negative GR co-chaperone FK binding protein 51 (FKBP51) in the VMH of male mice (Balsevich et al., 2014). Upregulated FKBP51 and reduced GR in the hypothalamus may be one mechanism leading to glucocorticoid resistance after consumption of an energy-dense diet.

The GR is not the only modulator of HPA axis output affected by (and affecting) food intake. CRF appears to have dual roles with respect to food intake. Central intracerebroventricular infusion of CRF can result in upregulation of pituitary POMC and concomitant weight loss or delayed weight gain in both normal weight (Buwalda, de Boer, Van Kalkeren, & Koolhaas, 1997; Hotta et al., 1991) and obese rats (Arase, Shargill, & Bray, 1989). The effects of CRF central infusion on weight also appear sex-dependent, reducing intake and promoting weight loss in male animals but not females (Rivest, Deshaies, & Richard, 1989). In contrast to CRF infusion, however, transgenic overexpression of CRF can promote increased food intake and weight gain (Dedic et al., 2012; Nakayama et al., 2011; Stenzel-Poore, Duncan, Rittenberg, Bakke, & Heinrichs, 1996), indicating a such as receptor downregulation or mediation of ACTH release by factors other than CRF, such as arginine vasopressin (Inui, 2000).

CRF can act on either of two receptors, CRF_1 or CRF_2, which are differentially sensitive to CRF and appear to play separate roles in feeding and stress regulation (Bale & Vale, 2004; Cullen, Ling, Foster, & Pelleymounter, 2001). In mice, CRF_1 receptor agonists urocortin 1 (UCN1) and stressin-A reduce food intake while increasing corticosterone, while urocortin 2 (UCN2) reduced food intake without an effect on the stress phenotype in a CRF_2 receptor-dependent mechanism (Fekete et al., 2011). The CRF_2 receptor system may be primarily responsible for anorexigenic effects of CRF or UCNs (Stengel & Tache, 2014) while the CRF_1 receptor may be responsible for the stress-induced increase in palatable food consumption or binge-eating (Koob, 2010; Parylak, Koob, & Zorrilla, 2011). In support of this role for the CRF_1 receptor, antagonism of central CRF_1 receptors in socially subordinate female rhesus macaques blocks the increase in palatable food consumption observed with access to a diverse dietary environment (C. J. Moore, Johnson, Higgins, Toufexis, & Wilson, 2015). Manipulation of a biological factor such as CRF or corticosterone can have diverse effects depending on the site of action and given different environmental exposures, such as diet.

1.3.2 Energetic stress impairs central glucocorticoid signaling In addition to the specific effects of diet, changes in energy availability, notably in the form of obesity and other metabolic dysfunction, can result in significantly altered central glucocorticoid signaling. Relative to women with more peripheral gluteofemoral fat distribution, viscerally obese women have elevated salivary cortisol after an ACTH stimulation test as well as attenuated salivary cortisol after a dexamethasone suppression test (Duclos et al., 2001), a pattern suggestive of HPA axis hypersensitivity. A similar pattern of central hypersensitivity has also been observed in men, as evening serum cortisol levels in men correlate negatively with waist-to-hip ratio (WHR), and a WHR of greater than one is associated with reduced serum cortisol in the dexamethasone suppression test (Ljung, Andersson, Bengtsson, Bjorntorp, & Marin, 1996). In adolescent girls, elevated BMI is associated with central hypersensitivity and depression, though such a link was not observed in adolescent boys (Dockray, Susman, & Dorn, 2009). However, the literature is

not entirely consistent with respect to central glucocorticoid hypersensitivity. Premenopausal adult women with visceral obesity have been observed to have increased morning ACTH pulsatility but reduced ACTH pulse amplitude without total changes in plasma cortisol relative to healthy controls, indicating disruption at the level of the pituitary (Pasquali et al., 1998). In addition, as previously mentioned, Type Two diabetes is linked to high basal cortisol and dexamethasone non-suppression, (Duong et al., 2012), and obese men have shown non-suppression of ACTH and cortisol after evening exposure to hydrocortisone (Jessop, Dallman, Fleming, & Lightman, 2001),in both cases indicative of glucocorticoid resistance, and not hypersensitivity.

Given the lack of clarity achieved by examining the effects of metabolic factors on glucocorticoid signaling at one time point, other studies have sought to examine glucocorticoid signaling patterns. In a study of the association between HPA axis activity and anthropometric measures in 284 51-year-old mean, reduced salivary cortisol diurnality, characterized by low morning cortisol and a blunted cortisol surge in response to a midday meal, was associated with a wide spectrum of negative cardiometabolic factors, including elevated body mass index (BMI), increased WHR, elevated fasting glucose and insulin, increased triglycerides and cholesterol, and elevated blood pressure (Bjorntorp & Rosmond, 1999). Further subdivisions of diurnal salivary cortisol and stress reactivity in this cohort of men indicated that three groups existed: one, with high diurnal variability, a typical cortisol response to food intake, and low stress reactivity; a second, with high diurnal variability, elevated cortisol after food intake, and high stress reactivity (approximately a third of the cohort); and the third group, with low diurnal variability, reduced response to food intake, and low stress reactivity (approximately a tenth of the cohort) (Bjorntorp & Rosmond, 2000). This last group with low plasticity has been termed a "burnout" condition that may follow prolonged stress. Indeed, this low plasticity is characteristic in those who have experienced prolonged severe stress (B. S. McEwen, 1999), in some cases of depression (Burke, Davis, Otte, & Mohr, 2005), and in some patients with PTSD (Meewisse, Reitsma, de Vries,

Gersons, & Olff, 2007). Thus, it appears that changes in energy availability can affect the pattern of central glucocorticoid signaling, and that this pattern may reflect the chronicity of disruption and correlate with alterations in the stress response.

1.3.3 Energetic stress alters glucocorticoid activity in peripheral tissues In addition to its multiple central effects, disrupted energy availability can profoundly alter peripheral actions of the HPA axis, primarily by changing glucocorticoid signaling in non-neural tissue. This change, in turn, may reflect back on central targets of the HPA axis by altering other neuroendocrine targets, inflammatory mediators, and the gut-brain axis (discussed subsequently). To understand the interplay between energetic stress and peripheral glucocorticoid signaling, one must first examine the baseline role of glucocorticoids in peripheral tissues. As reviewed in (Pasquali, Vicennati, Cacciari, & Pagotto, 2006) and (Carroll et al., 2011), glucocorticoids mobilize sources of energy to different tissues, specifically increasing lipolysis in adipose tissue and gluconeogenesis in liver while inhibiting glucose uptake and insulin receptor function. This has the end result of increasing post-absorptive and circulating glucose and free fatty acids. Additional actions of glucocorticoids include reducing calcium reabsorption; regulating gonadotrophic and growth hormone axes; immunosuppression; increasing contractility and vascular reactivity; and increasing appetite. In humans, active cortisol is synthesized from metabolism of cholesterol, and specifically through hydroxylation of 11-deoxycortisol by 11β-hydroxylase. Cortisol can then be inactivated by conversion to cortisone by 11β-hydroxysteroid dehydrogenase-2 (11βHSD2) and cortisone can be reconverted to cortisol by 11β-hydroxysteroid dehydrogenase-1 (11βHSD1). 11βHSD1 can be considered an amplifier of glucocorticoid action, and its action in liver, fat, and skeletal muscle, as well as neurons, may play an important role in the feedback of the HPA axis (reviewed in (Sandeep & Walker, 2001)).

Lipid accumulation and alterations in lipolytic pathways are associated with changes in glucocorticoid signaling, with tissue-specific variation in glucocorticoid-mediated effects (Joyner, Hutley, & Cameron, 2000; X. Xu, De Pergola, & Bjorntorp, 1990). Visceral adipose tissue has a

four times greater density of GR than subcutaneous adipose tissue, and typically has a greater activity of GR-mediated lipoprotein lipase (LPL) (S. B. Pedersen, Jonler, & Richelsen, 1994). Given the role of LPL in adipocyte differentiation, this is one mechanism whereby glucocorticoids preferentially promote accumulation of visceral fat (reviewed in (McCarty, 2001)). Sex hormones interact with glucocorticoids and LPL to differentially impact fat distribution as well; estrogen enhances activity in subcutaneous adipocytes while testosterone enhances activity in visceral fat, resulting in the sex differences observed in central obesity (Bjorntorp, 1997; Ramirez et al., 1997; Rebuffe-Scrive et al., 1987). Tissue-specific changes in 11βHSD1 expression and activity also appear to play a role in obesity-induced actions on glucocorticoid signaling. While obesity is associated with a reduction in liver 11βHSD1, it is linked with an increase in adipose tissue 11βHSD1 in both humans (Rask et al., 2002a) and animal models (Masuzaki et al., 2001). Transgenic overexpression of 11βHSD1 results in visceral obesity and insulin resistance (Wamil & Seckl, 2007). Conversely, 11βHSD1 knockout mice are resistant to hyperglycemia induced by either high-fat diet or psychosocial stress (Kotelevtsev et al., 1997). Moreover, 11βHSD1 may play a role in the resolution of inflammation; cytokines upregulate 11βHSD1 in adipocytes (Tomlinson et al., 2001), potentially as a counter-regulatory mechanism to induce the production of anti-inflammatory cortisol. However, this could theoretically result in a feed forward loop leading to upregulation of cortisol, which could enhance adipocyte differentiation, which may in turn result in elevations in adipokine production, and further enhancement of 11βHSD1 conversion of inactive cortisone to cortisol. Though the mechanisms remain to be elucidated, the current literature points to a role of visceral obesity resulting in elevated glucocorticoid actions, which have the potential to further disrupt HPA axis signaling.

1.3.4 Energetic stress induces hyperactivity of the sympathetic nervous system The other arm of the stress axis, the sympathetic nervous system (SNS), is also modulated by disruptions to energy availability. For example, SNS hyperactivity underlies the relationship

between weight and essential hypertension (Esler et al., 2001; Landsberg, 1986). The majority of essential hypertension risk can be explained by obesity in both men and women (Garrison, Kannel, Stokes, & Castelli, 1987). Obesity results in high rates of spillover of norepinephrine from both the heart and kidneys, which are likely attributable to increases in sympathetic nerve firing rates (Esler et al., 2001). Animal models have helped us to understand the pathophysiology of essential hypertension, including diet-induced obesity models (Dobrian, Davies, Prewitt, & Lauterio, 2000), the obese Zucker rat (Kurtz, Morris, & Pershadsingh, 1989), or the spontaneously hypertensive rat (Jacob et al., 1991). Increased sympathetic neural activity (SNA) is a common feature to these animal models of obesity (Rahmouni, Morgan, Morgan, Mark, & Haynes, 2005), and is associated with increased levels of circulating leptin and insulin alongside decreased levels of ghrelin and adiponectin, which may also contribute to SNA (Vaneckova et al., 2014). Recent work demonstrates that leptin mediates essential hypertension through its actions on hypothalamic proopiomelanocortin (POMC) neurons, which results in activation of melanocortin 4 receptors (MC4R) and stimulation of SNA (da Silva, do Carmo, & Hall, 2013). Leptin additionally mediates SNA in peripheral tissues, including the adrenal glands, kidneys, and muscle (Dunbar, Hu, & Lu, 1997). Selective leptin resistance may develop in obesity such that increased leptin fails to incite satiety and weight reduction, but continues to induce SNA (Mark, 2013).

This SNS hyperactivity in obesity may have significant crosstalk with the HPA axis. Normal sympathetic activation stimulates the adrenal cortex to secrete glucocorticoids, while HPA axis activity augments sympathetic effects, such as vasoconstriction for maintenance of blood pressure (Ulrich-Lai & Herman, 2009). SNS hyperactivity in obesity can result in an exacerbated response to acute stress or a CRF/AVP challenge, and can occur in conjunction with HPA axis hyperactivity (Pasquali et al., 1996). These changes in SNA can have important health outcomes, as autonomic imbalance, measured as reduced heart rate variability due to increased SNA in the context of vagal activity withdrawal, can result in increased risk of myocardial

infarction (MI) in obese patients (Karason, Molgaard, Wikstrand, & Sjostrom, 1999). Weight loss can reverse this imbalance in some cases and may be able to decrease risk of adverse cardiac outcomes (Karason et al., 1999). Intriguingly, similar changes in heart rate variability and increased risk of MI occur in the context of depression (Carney et al., 1995) and depressive symptoms increase the risk of mental-stress induced myocardial ischemia after a prior MI (J. Wei et al., 2014), though these risks can occur even controlling for metabolic risk factors. Taken together, disruptions to energy availability alter signaling of both axes of the stress response, and sympathetic hyperactivity may modulate the patterns of HPA axis activity observed in obesity, resulting in potentially fatal pathology.

1.3.5 Energetic stress disrupts signaling of neuroendocrine and neuropeptide factors that interact with the HPA axis The previous several sections have made evident that metabolic effects on the HPA axis are caused not only through direct interactions with components of the axis, but also through indirect mediators. For example, we have already discussed the manner in which adipocyte production of leptin can promote excess sympathetic nerve activity (da Silva et al., 2013), and hyperactive SNA can induce hyperactive HPA activity (Pasquali et al., 1996). Leptin is additionally an adipokine, or cytokine produced by adipose tissue, and the effects inflammatory factors on the HPA axis will be further discussed in the next section. However, several other neuroendocrine and neuropeptide factors play important modulatory roles impacting the HPA axis in the context of disrupted energy homeostasis.

One category of such factors includes the melanocortins and their receptors. The melanocortins are the group of ligands derived from the precursor POMC (ACTH, α, β, and γ-melanocyte-stimulating hormone (MSH)) that bind G-protein coupled receptors that activate adenylyl cyclase (Huszar et al., 1997). MC2R is the receptor for ACTH and is primarily expressed in adrenal cortex (Mountjoy, Robbins, Mortrud, & Cone, 1992), while MC3R and MC4R are neural melanocortin receptors activated by agouti-related protein (AgRP) and melanocyte-stimulating hormones. Both MC4R knockout mice and POMC knockout mice

display hyperphagia and weight gain (Zemel & Shi, 2000), and this pathway is believed to be the mechanism whereby the agouti gene induces obesity (Huszar et al., 1997). MC4R is also implicated in human obesity, as several mutations in the gene are associated with inherited forms of obesity (Farooqi et al., 2000). Given this relationship, there has been growing interest in the potential of MC4R agonists as potential weight loss aids; however, clinical trials have so far proven inefficacious due to lack of weight loss and non-serious adverse events (Krishna et al., 2009; Royalty, Konradsen, Eskerod, Wulff, & Hansen, 2014). Interestingly, rats heterozygous for a loss-of-function mutation in MC4R have a blunted response to stress in terms of plasma ACTH and corticosterone as well as reduced activation of the paraventricular nucleus of the hypothalamus and the medial amygdala (Ryan et al., 2014). Moreover, exposure to the intranasal MC4R antagonist HS014 prevents the development of anxiety- and depressive-like behaviors in rats (Serova, Laukova, Alaluf, & Sabban, 2013). Thus, the melanocortins and MC4R in particular appear to play a key role in regulating the bidirectional relationship between obesity and mood disorders that remains to be further explored.

Neuropeptide Y (NPY), a 36 amino acid residue peptide of the pancreatic polypeptide family, is another modulator of the relationship between metabolic dysfunction and mood. CNS administration of NPY results in a multi-fold increase in food intake in rats (Morley, Levine, Gosnell, Kneip, & Grace, 1987). NPY acts on hypocretin/orexin neurons in the arcuate nucleus to promote feeding, though intriguingly NPY seems to inhibit orexigenic hypocretin neurons while still inducing consumption (Fu, Acuna-Goycolea, & van den Pol, 2004). Diet-induced obesity reduces hypothalamic NPY (Levin, 1999; Lin, Storlien, & Huang, 2000), seemingly part of a negative feedback loop. NPY promotes HPA axis activity, as microinjections of NPY into the PVN can stimulate ACTH and corticosterone release (Wahlestedt et al., 1987). Conversely, corticosterone is necessary for NPY elicited food intake (Stanley, Lanthier, Chin, & Leibowitz, 1989). In spite of this HPA axis activation, NPY produces anxiolytic effects in rodent models, though its behavioral effects may be due to activity in the basolateral amygdala (Heilig et al.,

1993; Sajdyk et al., 2008). With respect to neuropsychiatric conditions, depression and PTSD are associated with reduced cerebrospinal NPY (Heilig et al., 2004). Moreover, anti-depressants (Heilig, Wahlestedt, Ekman, & Widerlov, 1988) increase hippocampal NPY, though hypothalamic expression of NPY in depression is less well understood. Collectively, however, it is possible that obesity-induced alterations in NPY signaling contribute to the depressive- and anxiety-like behavioral changes seen in obesity.

Though a complete examination of neuropeptide and neuroendocrine factors that are altered in metabolic dysfunction is beyond the scope of this review, it should additionally be noted that factors such as thyroid hormone and growth hormone have impaired signaling in obese states (Douyon & Schteingart, 2002), and these same factors are also inhibited with hypothalamic arousal (Chrousos & Gold, 1992). Furthermore, altered signaling of both thyroid (Fountoulakis, Iacovides, Grammaticos, St Kaprinis, & Bech, 2004; Joffe & Marriott, 2000; Musselman & Nemeroff, 1996) and growth hormone (Linkowski et al., 1994) can be depressogenic. The changes in melanocortins, NPY, and additional neuropeptide and neuroendocrine factors may represent a broader state of hypothalamic remodeling that occurs in response to disruptions of energetic homeostasis to alter HPA axis activity and behavior.

1.3.6 Energetic stress alters the HPA axis response to inflammatory stimuli

Inflammatory stimuli, whether bacterial or viral challenges or even psychosocial stressors, are known to activate the HPA axis to potentiate hypothalamic secretion of CRF and AVP as well as adrenal glucocorticoid secretion via the actions of cytokines and chemokines (Charmandari, Tsigos, & Chrousos, 2005; Pace & Miller, 2009). This HPA axis stimulation is evolutionarily adaptive, as the GR functions as an immune modulator not only through its activity as a transcription factor but also through direct protein-protein interactions with other transcription factors such as NfκB and AP-1 (Pariante & Lightman, 2008). However, chronic inflammation can result in long-term disruption of the HPA axis including impaired glucocorticoid negative feedback.

Changes energy availability in states such as obesity and diabetes (Arkan et al., 2005; Herder et al., 2007; Hotamisligil, 2005; M. I. Schmidt et al., 1999), as well as in malnutrition or anorexia (Kalantar-Zadeh, Kopple, Humphreys, & Block, 2004; Nakai, Hamagaki, Takagi, Taniguchi, & Kurimoto, 1999), are associated with chronic inflammation. Indeed, when the International Diabetes Federation introduced the new worldwide definition for metabolic syndrome, it highlighted the proinflammatory state as a key area of research to determine the predictive power of such factors as C-reactive protein (CRP), tumor necrosis factor-α (TNF), and interleukin-6 (IL6) for cardiovascular disease or diabetes (Alberti, Zimmet, & Shaw, 2006; IDF, 2006). These recommendations come from both clinical research that show consistent elevations in circulating CRP, TNF, and IL6 in obesity and/or diabetes (Brunner et al., 2002; Lamers et al., 2013; M. I. Schmidt et al., 1999). At least 16 different adipokines, or chemokines or cytokines secreted by adipose tissue, are over-secreted in human obesity, including IL1, IL6, IL8, monocyte-chemotactic protein-1 (MCP-1), and leptin (Maury et al., 2007). Basic research supports the association between metabolic dysfunction and inflammation and has been able to directly link increased adipose tissue expression of TNF in particular to development of insulin resistance via TNF-mediated serine phosphorylation of insulin related substrate-1 (IRS1) (Hotamisligil, Shargill, & Spiegelman, 1993).

This research linking metabolic dysfunction to inflammation has arisen from a growing literature demonstrating that adipose tissue is not only metabolically active, but moreover a major secretor of bioactive peptides and proteins that play a central role in energy balance and immunity (Maury & Brichard, 2010). The adipokines adiponectin, leptin, and resistin are primarily produced in adipose tissue and changes in these factors are associated with metabolic dysfunction**.** Adiponectin, commonly reduced in obesity, interacts with its receptors to suppress the nuclear factor κB-dependent synthesis of TNF and interferon-γ (IFNγ) while promoting production of anti-inflammatory mediators including IL-10 and IL-1Ra (Tilg & Moschen, 2006). Leptin's actions on the sympathetic nervous system have already been discussed, but it

additionally promotes production of the pro-inflammatory cytokines TNF, IL-6, and IL-12 as well as reactive oxygen species through some of the same molecular pathways involved in SNA, included MAPK, ERK, and STAT3 signaling (Tilg & Moschen, 2006). Resistin is less well understood. Diet-induced models of obesity increase resistin, which increases expression of adhesion molecules, and antibody-mediated neutralization of resistin enhances blood sugar regulation, potentially linking diabetes and obesity mechanistically (Steppan et al., 2001).

In terms of understanding the relevance of metabolically-mediated immune alterations to mental health, Lamers et al (Lamers et al., 2013) suggest that one might be able to use biological correlates in metabolic, inflammatory, and endocrine systems to differentiate between depressive subtypes. In their study of 122 atypical depressed patients, 111 melancholic depressed patients, and 543 controls, the authors found that atypical depressed patients had increased plasma levels of the inflammatory markers CRP, TNF, and IL-6 along with a negative cardiometabolic profile characterized by high BMI, large waist circumference, elevated triglycerides, and elevated fasting glucose, and a concomitant reduction in the diurnal cortisol slope (Lamers et al., 2013), perhaps indicative of the HPA axis burnout described earlier (Bjorntorp & Rosmond, 2000; B. S. McEwen, 1998; Meewisse et al., 2007). Conversely, the melancholic depressed patients showed reduced BMI and increased area under the curve for diurnal cortisol and an increased diurnal cortisol slope, indicating HPA axis hyperactivity (Lamers et al., 2013). One limitation of the study is that due to the cross-sectional nature of the study, one cannot draw conclusions about the causal or directional nature of these findings; nonetheless, these findings do point to important relationships among metabolic, inflammatory, and endocrine factors that can impact behavioral phenotype. Taken together, however, it is clear that changes in energy availability are linked to chronic low-grade inflammation, and such inflammation has negative repercussions on HPA axis function and mental health.

1.3.7 Energetic stress modulates the gut-brain axis to dysregulate the HPA axis Though a recent resurgence of attention has brought it to the forefront of stress physiology, the reciprocal

interaction between the brain and the gastrointestinal tract has been described since the mid-nineteenth and early twentieth century through the work of classical physiologists including Claude Bernard, Ivan Pavlov, William James, Carl Lange, and Walter Cannon (Cryan & Dinan, 2012). In "The Emotions" (Lange & James, 1922), James and Lange developed the "James-Lange theory of emotion" arguing that emotions followed the visceral reaction to a stimulus. To explain differences in susceptibility to emotional expression, James wrote, "the visceral and organic part of the expression can be suppressed in some men, but not in others, and on this it is probably that the chief part of the felt emotion depends" (p. 116) (Lange & James, 1922). Cannon alternatively refuted this primacy of the viscera, arguing that the brain principally regulated such reactions due to factors such as the slow timing of the visceral response and the similarity of visceral reactions across a wide spectrum of emotions (Cannon, 1927).

We now recognize the bidirectional nature of this interaction and thus term it the "gut-brain axis" (Cryan & Dinan, 2012). During homeostasis, the brain signals to the gut through both branches of the autonomic nervous system; the HPA axis; the sympatho-adrenal axis; and descending monoaminergic signals (Mayer, 2011). The gut, in turn, signals to the brain through primary afferent neurons; immune cells, and enteroendocrine cells (Mayer, 2011). Enteroendocrine cells can communicate to the hypothalamus via either endocrine or paracrine (often vagal) signaling. Primary vagal and spinal afferent signals are integrated via the NTS and lamina I and subsequently through the thalamus and insula, and can communicate nutritional and inflammatory state information to cortical regions (Mayer, 2011).

Changes in energetic state can perturb this homeostatic relationship. A well-characterized example of this perturbation occurs through the CRF system in irritable bowel syndrome (IBS). Peripheral CRF and urocortin act via CRFR1 and CRFR2 to inhibit gastric emptying and stimulate colonic transit and defecation, while urocortin 2 and 3 act through CRFR2 only to inhibit gastric emptying (Martinez, Wang, Rivier, Vale, & Tache, 2002). Patients with IBS exhibit exacerbated intestinal motility, enhanced mast cell response, and increased gut

permeability in response to systemic CRF administration (Tache & Million, 2015). In addition, IBS patients have lower basal CRF relative to healthy controls but an enhanced CRF and ACTH response to mental stress (Posserud et al., 2004). Conversely, IBS patients have higher basal norepinephrine but a decreased norepinephrine response to stress (Posserud et al., 2004). In animal models, acute stress activates both peripheral and central CRFR1 to stimulate gastric emptying (Tache & Perdue, 2004), and early life stress increases both gut permeability and this CRF-mediated response to acute stress (Soderholm et al., 2002). Anxiety and depression are highly comorbid with IBS, and the enhanced neuroendocrine responses to stress may play a role in this comorbidity (Mayer, Naliboff, Chang, & Coutinho, 2001).

More recent literature has implicated the gut microbiome in both metabolic dysfunction and mood disorders. The human gastrointestinal tract comprises between 10^{13} and 10^{14} microorganisms and more than 100 times the number of genes in the human genome (Gill et al., 2006). The mammalian microbiome is composed primarily of the gram-positive *Bacteroidetes* and *Proteobacteria* as well as the gram-negative *Firmicutes* and *Actinobacteria* phyla with lower abundance of *Fusobacteria,* and *Verrucomicrobia* (Eckburg et al., 2005; Gill et al., 2006; Tilg & Kaser, 2011). As humans are born with a relatively sterile gut, the microbiome is developed throughout childhood and into adulthood (Koenig et al., 2011). This development can be influenced by such factors as diet, illness, antibiotic treatment, and other environmental exposures. Notably, a high-fat diet can shift the mouse microbiome towards a greater predominance of *Firmicutes,* and this shift is both reversible (Turnbaugh, Backhed, Fulton, & Gordon, 2008) and can occur in the absence of obesity (Hildebrandt 2009). In the absence of a high-fat diet, obesity in ob/ob mice is also associated with an increase in *Firmicutes* with a relative decrease in *Bacteroidetes* compared with lean mice (Ley et al., 2005). The microbiome in obese mice enables greater energy harvest than in lean mice, and transfer of caecal microbiota from ob/ob mice to germ-free mice is sufficient to produce an obese phenotype (Turnbaugh et al., 2008). In humans, obesity is associated with a decrease in phylogenetic diversity of the

microbiome, with approximately 75% of obesity-associated genes derived from *Actinobacteria* (vs. 0% of lean-associated genes) and 25% from *Firmicutes* (Turnbaugh et al., 2009). In contrast, 42% of lean-associated genes in humans derive from *Bacteroidetes,* compared with 0% of obesity-associated genes (Turnbaugh et al., 2009). Though research is in the nascent stage and evidence is most convincing for its efficacy to treat inflammatory bowel disease (J. L. Anderson, Edney, & Whelan, 2012), fecal microbiota transplantation has shown some promise in increasing insulin sensitivity in obese men (Vrieze et al., 2012). While much work remains to be done with respect to the regulatory role of diet, genetic background, and other environmental factors, it is clear that the gut microbiome has a strong influence on metabolic phenotype.

In addition to its impact on energy availability, the microbiome has a bidirectional relationship with the development of the stress response and behavioral outcomes. Stress can alter the microbiome, like high-fat diet, to decreases the relative abundance of *Bacteroidetes* and increases circulating levels of the inflammatory cytokines IL-6 and MCP-1 (Bailey 2011). These effects are blocked by administration of antibiotics, indicating the necessity of microbiota in moderating the response to social disruption stress (Bailey 2011). However, just as stress can alter the microbiotic composition, so too can the composition of the microbiome determine behavior and the stress response. Germ-free (GF) mice exhibit reduced anxiety-like behaviors relative to specific-pathogen free (SPF) mice with normal gut microbiota, and colonization of adult germ-free mice fails to reverse their behavior (Diaz Heijtz et al., 2011). However, offspring of GF mice exposed to gut microbiota exhibit anxiety-like behavior in the elevated plus maze similar to SPF mice(Diaz Heijtz et al., 2011), indicating a potential critical period for the microbiotic influence on behavior. This critical period of microbiotic influence has also been demonstrated with respect to HPA axis development. Intriguingly in light of their behavior, GF mice exhibit greater ACTH and corticosterone responses to restraint stress than SPF mice (Sudo et al 2004). Colonization of GF mice with *Bifidobacterium infantis* reduces this stress response while *Escherichia coli* enhances it. Colonization with gut microbiota from SPF mice can also

reduce this enhancement in the HPA axis response, and colonization earlier in development is more effective (Sudo et al., 2004). Though the exact mechanism remains unknown, changes in the gut microbiome are associated with alterations in the immune system, BDNF, and GABA-ergic signaling, which may all contribute to microbiotic-mediated regulation of the HPA axis (J. A. Foster & McVey Neufeld, 2013). Continued efforts using probiotics and fecal transplants to combat obesity and alter the HPA axis and stress response will shed more light on the relative importance of these proposed mechanisms (Dinan, Stanton, & Cryan, 2013). Collectively with our knowledge of the gut microbiome's role in regulating energy balance, such research may result in novel approached to target disorders associated with both metabolic and mental health outcomes.

1.4 Discussion

Throughout this review, we have discussed the potential for perturbations in energy availability (primarily in obesity and diabetes) to alter the stress response. Though we have analyzed this relationship between energy availability and the stress response and mechanisms for disruption in separate sections, we must heed Claude Bernard's advice in drawing our final conclusions with respect to the effects on the whole organism. Clearly, energy balance is maintained through multiple organ systems, not only through actions of the hypothalamus. These systems include the digestive system, the immune system, the musculoskeletal system (including adipose tissue), the endocrine system, and several arms of the nervous system. Two arms, in turn, regulate the stress response: the swift-acting sympathetic nervous system, and the longer acting HPA axis. Given the wealth of literature examining the role of the HPA axis in energy homeostasis (Dallman et al., 2005; Rosmond, 2005), the bulk of this review has focused on interactions of metabolic dysfunction with this axis.

As summarized in Table 1.3, we began by exploring three potential mechanisms relating directly to the HPA axis that mediate the relationship between changes in energy availability and the stress response. First, diet has a reciprocal relationship with the HPA axis, as palatable diet can both dampen or enhance reactivity while HPA axis activity can alternatively promote or inhibit consumption of palatable or non-palatable food (**Section 1.3.1**). However, this relationship is altered dependent on the energetic status of the individual, and metabolic dysfunction can impair this balance. Next, such energetic stress can not only alter central glucocorticoid signaling at a single time point, but they can shift whole signaling patterns (**Section 1.3.2**). Changes in diurnal cortisol variability are linked to changes in the cortisol response to food as well as stress reactivity, and different patterns may represent different stages of allostatic load to "burnout" (B. S. McEwen, 1998; Rosmond et al., 2000). Peripheral glucocorticoid signaling is also altered by disrupted energy homeostasis, resulting in the potential for glucocorticoid-mediated shifts toward fuel storage in visceral adipose tissue, which can in turn feedback to affect central HPA axis mechanisms (**Section 1.3.3**).

We subsequently examined several other systems that mediate the relationship between energy availability and the stress response. Energetic stress results in SNS hyperactivity, changing the crosstalk between the SNS and the HPA axis (**Section 1.3.4**). Further, multiple neuroendocrine and neuropeptide factors in addition to glucocorticoids and the SNS respond to energetic stressors, particularly POMC/melanocortins and NPY. In turn, this altered neuropeptide and neuroendocrine signaling can promote excessive ACTH and glucocorticoid stimulation (**Section 1.3.5**). Energetic stress also stimulates low-grade inflammation through some of these neuroendocrine factors, such as leptin, as well as other through other adipokines and cytokines. This inflammation can subsequently have negative repercussions on the HPA axis and mental health (**Section 1.3.6**).

Collectively, these multiple mechanisms represent areas of future research and target areas for potential therapeutics. The need for better therapies is high; with respect to depression,

50-60% of patients fail to achieve an adequate response to antidepressant treatment (Fava, 2003). Indeed, treatment of mental health disorders may be complicated by comorbid disorders such as obesity or diabetes (Fava, 2003), and second-order treatments such as atypical antipsychotics can have negative repercussions on metabolism (Nasrallah, 2003). If direct links can be established between failures in energy homeostasis and mental health outcomes, then targeting the disruption in these links is the next step to improved therapy. For example, changes dietary modulation, such as adopting a "Mediterranean" diet, have been proposed to assist in mental health therapy (Psaltopoulou et al., 2013). The "Mediterranean" diet may be effective in the context of depression because of its high content of omega-3 fatty acids, which have been shown to alleviate depressive symptoms in some patients (Freeman & Rapaport, 2011). Interestingly, omega-3 fatty acid therapy may be most effective in patients with a higher inflammatory profile, linking the role of diet to inflammation and mental health (Rapaport et al., 2015). L-methylfolate (Papakostas et al., 2012) is another therapy designed as adjuvant to selective serotonin reuptake inhibitors (SSRIs) to address the relationship deficit in micronutrient status and mood. In addition, deep brain stimulation (DBS) in the subcallosal cingulate gyrus (Lozano et al., 2008) as well as in the nucleus accumbens (Bewernick et al., 2010) has been recently demonstrated as an effective therapy for treatment resistant depression. Interestingly, DBS in the nucleus accumbens has also been proposed as a therapy for obesity to address changes in hedonic drive (Halpern et al., 2008).

In moving forward, researchers should pay special attention to the potential sex differences that can affect both energy availability and stress reactivity. Body morphology differs significantly between males and females, with females typically carrying greater gluteofemoral and subcutaneous fat (Lovejoy, Sainsbury, & Stock Conference Working, 2009; Westerbacka et al., 2004). While there is generally a slightly lower population prevalence of obesity in men, obese men are at a higher risk of obesity-related chronic disease, due, in part, to the visceral accumulation of fat (Lovejoy et al., 2009). Women generally have a better cholesterol profile and

greater insulin sensitivity than men (Cnop et al., 2003), though hormonal status (such as menopause) may moderate this effect (Lovejoy et al., 2009). At the same time, women are at a roughly two-fold risk of lifetime depression (Kessler et al., 2003). Men, however, are not immune to mental health disorders; for example, they are two to three times more likely than women to abuse drugs (Becker & Hu, 2008). In this review, significant sex differences were noted in the effects of central CRF on weight (Rivest et al., 1989) as well as in the actions of LPL and glucocorticoids on adipose tissue (Rebuffe-Scrive, Andersson, Olbe, & Bjorntorp, 1989). In addition, several disorders that alter metabolism, such as IBS (Saito, Schoenfeld, & Locke, 2002) and autoimmune disorders like Hashimoto's and Graves' Disease (Whitacre, 2001), affect women substantially more than men. Greater research is needed to understand the specifics of how these sex differences interact with sex differences in mental health outcomes, and to determine if these potential relationships offer new avenues for therapies.

The role that development plays in regulating the relationship between energy availability and the stress response is also understudied. Developmental growth must by definition alter energy homeostasis, as the organism is in a state of flux. The current obesity epidemic is not limited to adults, as today over 20% of American adolescents are obese (Ogden, Carroll, Curtin, Lamb, & Flegal, 2010; Ogden et al., 2014) and Type II diabetes' rates are increasing among youth (Nadeau & Dabelea, 2008). Adolescence appears to be a "critical period" for determining energy homeostasis; evidence shows that high BMI during adolescence predicts overweight and obesity in adulthood; for example, the probability that a15-year-old with a BMI at the 95th percentile will be overweight at age 35 is 0.93 and, if male, the probability that he will be obese is 0.50 or, if female, 0.60 (Guo, Wu, Chumlea, & Roche, 2002). The concept of "critical periods" of development with respect to stress reactivity has also been examined across developmental periods, including both prenatal (Pankevich, Mueller, Brockel, & Bale, 2009; Tamashiro, Terrillion, Hyun, Koenig, & Moran, 2009) and adolescent (Bourke & Neigh, 2011; McCormick et al., 2013) periods. Adolescence may represent a critical period for crosstalk between energetic

regulatory systems, the HPA axis, and the hypothalamic-pituitary-gonadal axis, as each of these systems undergo extensive change during adolescence. Expanding research to include these key developmental stages when examining energy availability and stress reactivity will be essential toward understanding the emergence of a disrupted relationship and developing targeted therapies.

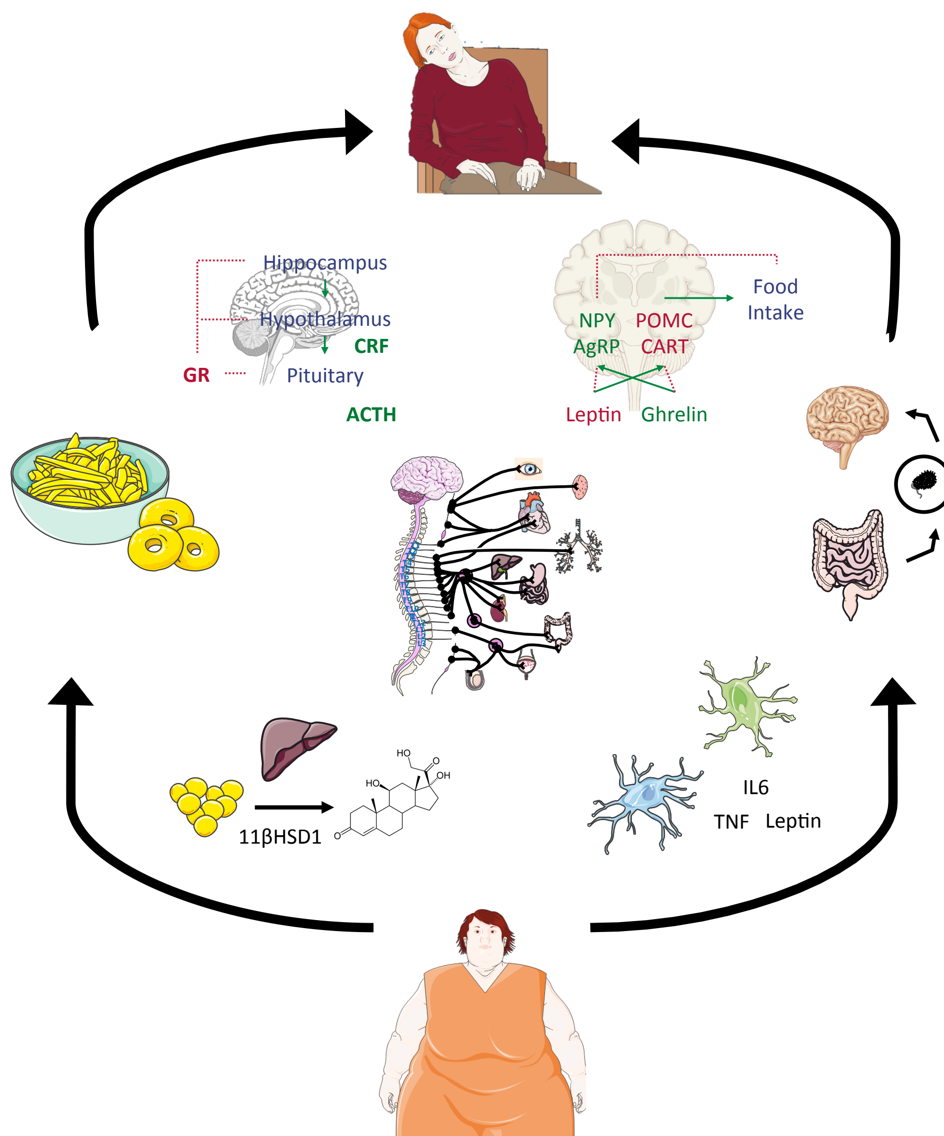
Of course, the best cure is prevention. Understanding the role of modifiable risk factors in energy availability and stress reactivity could ultimately lead to better policies and prevention strategies that might reduce the burden of metabolic dysfunction and mental health disorders from the start. One study examining risk factors associated with myocardial infarction across 52 countries determined that dietary patterns, abdominal obesity, regular physical activity, and psychosocial factors (including depression, locus of control and perceived stress) were among the potentially modifiable factors that could affect infarction risk (Yusuf et al., 2004). In terms of modifiable risk factors affecting obesity, a long debate has arisen between the relative roles of diet and physical activity. While both undoubtedly play a role, recent evidence examining long term trends in physical activity (Luke & Cooper, 2013) as well as differences in energy expenditure between individuals in highly industrialized vs. less industrialized countries (Luke & Cooper, 2013) indicates that physical activity may be less effective at preventing obesity than previously thought.

As for diet, nutritional experts differ in their recommended ideal dietary composition. The most recent nutritional guidelines from the United States Department of Agriculture and the Department of Human Health and Services called for significant limitations to dietary added sugars. While multiple dietary patterns, from low-carbohydrate to low-protein, can be effective at inducing weight loss in the short term with mixed results beyond one year (USDA), particular macronutrients may have specific effects on other aspects of metabolism or stress reactivity. For example, carbohydrates and proteins or fats elicit opposing effects on HPA axis reactivity in women with different body morphologies (Vicennati et al., 2002). Simple sugars, and fructose in

particular, are known to induce a spike in plasma corticosterone in rodent models (Brindley, Cooling, Glenny, Burditt, & McKechnie, 1981; Brindley, Saxton, Shahidullah, & Armstrong, 1985). However, there is a dearth of research into the specific effects of macro- and micronutrient status on brain function. Bridging the gap between nutritional research and the neuroscientific understanding of the relationship between energy availability and the stress response can lead to novel strategies for prevention to guide policy-makers and clinicians.

In conclusion, changes to energy availability induce energetic stress, which alter the stress response through a multifaceted approach. Energetic stress disrupts central and peripheral glucocorticoid signaling; promotes hyperactivity of the sympathetic nervous system; changes neuroendocrine and neuropeptide signaling; alters inflammatory responses; and perturbs the gut-brain axis. Greater research is needed to clarify the roles that sex and sex hormones, as well as developmental status, play in modulating these effects. Addressing these areas of research will provide new avenues for potential therapy. However, by researching the effects of modifiable risk factors, such as diet, we may be able to circumvent the need for such therapy by promoting prevention.

Figure 1.1



Disruptions to Energy homeostasis Alter the Stress Response through Multiple Mechanisms. States of disrupted energy availability, such as obesity, can result in an impaired stress response in seven key areas: macronutrient-induced alterations in glucocorticoid function; changes in central glucocorticoid signaling; changes in peripheral glucocorticoid signaling; alterations in sympathetic nervous system drive; modulation of neuroendocrine and neuropeptide signaling; and impairments in inflammatory response.

Table 1.1.

Nucleus	Location in Rat	Function
Arcuate Nucleus (ARC)	Circumventricular organ at the base of the third ventricle and above the median eminence	Allows entry of insulin and leptin; neurons express NPY, AgRP, and POMC
Paraventricular nucleus (PVN)	Superiorly located by the third ventricle, anterior to DMH	Receives convergence of NPY and orexin neurons
Ventromedial nucleus of the hypothalamus (VMH)	Superior to the ARC, inferior to DMH, and lateral to third ventricle	Responsive to leptin; plays a key role in inhibition of feeding
Dorsomedial nucleus of the hypothalamus (DMH)	Superior to VMH, posterior to PVN, lateral to third ventricle	Connects extensively with other hypothalamic nuclei; plays an integrative role in processing information
Lateral hypothalamus (LH)	Lateral to PVN, VMH; anteriorly located	Responsive to hypoglycemia, NPY; neurons express MCH, orexin

Hypothalamic Nuclei. Listed are the hypothalamic nuclei principally involved in regulation of energy homeostasis along with key functional attributes.

Table 1.2. Neuroendocrine and Neuropeptide Regulators of Energy Homeostasis

Factor	Effect on Feeding	Production Location	Receptor
Agouti-Related Peptide (AgRP)	Simulate	ARC	Melanocortin Receptors 3 & 4 (MC3R, MC4R) inverse agonist
Dopamine*	Stimulate	VMH, LH, Ventral tegmental area (VTA), striatum, additional regions	Dopamine Receptor 1 (D1R) in VMH
Dynorphin	Stimulate	LH, PVN, DMH	κ-Opioid Receptor
Galanin	Stimulate	ARC, DMH, median eminence, pituitary	GALR
γ-Aminobutyric acid	Stimulate	Widespread	$GABA_A$ Receptor
Ghrelin	Stimulate	Ghrelin cells in stomach and small intestine	Growth hormone secretagogue receptor
Glutamate	Stimulate	Widespread	N-Methyl-$_D$-Aspartate Receptor (NMDAR)
Melanin-Concentrating Hormone (MCH)	Stimulate	LH	Somatostatin-like Cloned Receptor-1
Orexin-A and B	Stimulate	DMH; LH; perifornical nucleus	OX1R and OX2R
Neuropeptide Y (NPY)	Simulate	ARC, PVN, supraoptic nucleus, suprachiasmatic nucleus (SCN)	NPY5R (primarily)
α-Melanocyte-Stimulating Hormone (αMSH)	Inhibit	In ARC and nucleus of the solitary tract (NTS) from proopiomelanocortin (POMC)	Melanocortin Receptors, primarily MC4R, but also MC1R, MC3R
Calcitonin-Gene Related Peptide	Inhibit	PVN, supraoptic nucleus, infundibular nucleus, motor neurons	Calcitonin-receptor like receptor
Cholecystokinin	Inhibit	Gut; Intestinal I cells	CCKBR
Cocaine and Amphetamine Related Peptide (CART)	Inhibit	ARC, PVN, LH, DMH, VMH, ventral striatum, basolateral amygdala; additional regions	Not fully characterized
Corticotropin Releasing Factor (CRF)	Inhibit	PVN; some peripheral production	CRFR1 and CRFR2
Dopamine*	Inhibit	VMH, LH, Ventral tegmental area (VTA), striatum, additional regions	D2R
Glucagon-like peptide-1 (GLP-1)	Inhibit	Gut; Intestinal L cells	GLP-1
Insulin	Inhibit	Pancreatic β cells	Insulin Receptor
Leptin	Inhibit	Adipose Tissue	Leptin Receptor (OBR)
Serotonin (5HT)	Inhibit	PVN, VMH, SCN; additional regions	5HT1R, 5HT2R
Somatostatin	Inhibit	VMH; stomach, intestine, pancreatic δ cells	SSTR3, SSTR4
Thyrotrophic-Releasing Hormone (TRH)	Inhibit	PVN	TRHR

(De Vry & Schreiber, 2000; Rondini, Baddini, Sousa, Bittencourt, & Elias, 2004; Williams et al., 2001; Williams et al., 2000)

Neuroendocrine and Neuropeptide Regulators of Energy Homeostasis Listed are notable neuroendocrine and neuropeptide regulators of energy homeostasis. *Note the dual listing of dopamine due to site- and receptor-specific actions.

Table 1.3

Feature	Effects with Respect to the HPA Axis and the Stress Response
Dietary Imbalance	Visceral obesity blunts the cortisol response post-ingestion of protein and lipids, but increases the response post-ingestion of carbohydrates (Vicennati et al., 2002) Excess fat or sugar consumption increases palatable food consumption after stress (36, 37) and blunts the glucocorticoid response to stress (M. T. Foster et al., 2009; Pecoraro et al., 2004; Tryon et al., 2015) OR Excess fat or sugar consumption elevates the glucocorticoid response to stress (Balsevich et al., 2014; Duong et al., 2012; Michopoulos et al., 2012; S. Sharma et al., 2013)
Central Glucocorticoid Signaling	Visceral obesity is linked with HPA axis hypersensitivity (Dockray et al., 2009; Duclos et al., 2001; Ljung et al., 1996) OR Visceral obesity is linked to glucocorticoid resistance (Duong et al., 2012; Jessop et al., 2001) Negative cardiometabolic profile is associated with reduced cortisol variability, low morning cortisol, and blunted cortisol response to midday meal (Bjorntorp & Rosmond, 1999)
Peripheral Glucocorticoid Signaling	Testosterone enhances lipoprotein lipase activity in visceral fat (Bjorntorp, 1997; Ramirez et al., 1997; Rebuffe-Scrive et al., 1987). Obesity increases adipose 11β-hydroxysteroid dehydrogenase-1 (11βHSD1) (Masuzaki et al., 2001; Rask et al., 2002a), which converts inactive cortisone to active glucocorticoids Increased 11βHSD1 may increase insulin resistance (Kotelevtsev et al., 1997; Wamil & Seckl, 2007) and promote inflammation (Tomlinson et al., 2001)
Sympathetic Nervous System (SNS)	SNS hyperactivity links obesity to essential hypertension (Dobrian et al., 2000; Esler et al., 2001; Garrison et al., 1987; Jacob et al., 1991; Kurtz et al., 1989; Landsberg, 1986; Rahmouni et al., 2005) Obesity-associated SNS hyperactivity may be caused by increased circulating leptin and insulin alongside reduced ghrelin and adiponectin (Vaneckova et al., 2014) Leptin resistance fails to incite satiety but continues to promote SNS activity (Mark, 2013); SNS hyperactivity stimulates the adrenal cortex and can exacerbate the response to stress (Pasquali et al., 1996)
Additional Neuroendocrine and Neuropeptide Factors	MC4R knockout mice are hyperphagic (Zemel & Shi, 2000) and have a blunted response to stress (Ryan et al., 2014) Diet-induced obesity reduces NPY, which promotes feeding (Levin, 1999; Lin et al., 2000); NPY is anxyiolytic in rodent models (Heilig et al., 1993; Sajdyk et al., 2008) though glucocorticoids activate NPY to elicit food intake (Stanley et al., 1989) Obesity alters thyroid and growth hormone signaling (Douyon & Schteingart, 2002), and which can be depressogenic (Fountoulakis et al., 2004; Joffe & Marriott, 2000; Musselman & Nemeroff, 1996) (Linkowski et al., 1994)
Inflammation	Obesity or diabetes (Arkan et al., 2005; Herder et al., 2007; Hotamisligil, 2005; M. I. Schmidt et al., 1999) as well as malnutrition or anorexia (Kalantar-Zadeh et al., 2004; Nakai et al., 1999) are proinflammatory and elevate CRP, TNF, and IL6 At least 16 different adipokines are oversecreted in obesity (Maury et al., 2007) Increased adipose TNF is directly linked to insulin resistance (Hotamisligil et al., 1993) Atypical depression is linked to a negative cardiometabolic profile, altered diurnal cortisol, and increased CRP, TNF, and IL6 (Lamers et al., 2013)
Gut-Brain Axis	IBS patients have lower basal CRF but increased CRF response to stress (Posserud et al., 2004) High-fat diet (Turnbaugh et al., 2008) and obesity (Hildebrandt et al., 2009) increase gut *Firmicutes* and reduce gut *Bacteroidetes* in rodents; In humans, obesity reduces phylogenetic diversity of the gut (Turnbaugh et al., 2009) Stress also alters the mouse gut microbiome and reduces Bacteroidetes (Bailey et al., 2011) and exposure of germ-free mice to gut microbiota induces anxiety-like behavior (Diaz Heijtz et al., 2011)

Effects of Energetic Stress on the HPA Axis and Stress Response The primary effects of energetic effects on each of the seven systems discussed are listed.

PART ONE:

EFFECTS OF STRESS OF A MARKER OF CEREBRAL ENERGY METABOLISM

Chapter Two

Psychosocial and Inflammatory Stressors Modulate Regional Glucose Transporter Expression in a Sexually-Dimorphic Manner

Adapted and Expanded from:

Kelly SD, Harrell CS, Neigh GN. Chronic stress modulates regional cerebral glucose transporter expression in an age-specific and sexually-dimorphic manner. *Physiology & Behavior.* 2014;126:39-49.

Pyter LM, Kelly SD, Harrell CS, Neigh GN. Sex differences in the effects of adolescent stress on adult brain inflammatory markers in rats. *Brain, Behavior, and Immunity.* 2013;30:88-94.

2.0 Abstract

Facilitative glucose transporters (GLUT) mediate glucose uptake across the blood-brain-barrier into neurons and glia. Deficits in specific cerebral GLUT isoforms are linked to developmental and neurological dysfunction, but less is known about the range of variation in cerebral GLUT expression in normal conditions and the effects of environmental influences on cerebral GLUT expression. Knowing that puberty is a time of increased cerebral plasticity, metabolic demand, and shifts in hormonal balance for males and females, we first assessed gene expression of five GLUT subtypes in four brain regions in male and female adolescent and adult Wistar rats. The data indicated that sex differences in GLUT expression were most profound in the hypothalamus, and the transition from adolescence to adulthood had the most effect on GLUT expression in the hippocampus. Next, given the substantial energetic demands during adolescence and prior demonstrations of the adverse effects of adolescent stress, we determined the extent to which chronic stress altered GLUT expression in males and females in both adolescence and adulthood. Chronic stress significantly altered cerebral GLUT expression in

males and females throughout both developmental stages but in a sexually dimorphic and brain region-specific manner. In addition, chronic adolescent stress significantly altered the effects of an inflammatory stimulus on GLUT expression in adulthood in a sex-dependent manner. Collectively, our data demonstrate that cerebral GLUTs are expressed differentially based on brain region, sex, age, and stress exposure. These results suggest that developmental and environmental factors influence GLUT expression in multiple brain regions. Given the importance of appropriate metabolic balance within the brain, further assessment of the functional implications of life stage and environmentally-induced changes in GLUTs are warranted.

2.1 Introduction

Adequate glucose transport is essential to brain function and survival. The adult brain accounts for 20% of total resting oxygen consumption in humans (Attwell & Laughlin, 2001; Mink, Blumenschine, & Adams, 1981; Sokoloff, 1989) and 4-6% in rats (Mink et al., 1981), which is almost entirely utilized for the oxidation of carbohydrates (Sokoloff, 1960). Regulating glucose transport throughout the various demands placed upon the brain and body is necessary for maintaining energy homeostasis and supporting brain development (Vannucci, 1994).

While the cerebral metabolic demand is substantial in the adult, the metabolic rate in children is much higher, calculating to as much as 50% of whole-body glucose utilization by the developing human brain (Sokoloff, 1960). Regulating of brain glucose utilization occurs primarily through glucose transporters (protein symbol GLUT, gene symbol *Slc2a*) (Simpson, Carruthers, & Vannucci, 2007). These transporters facilitate the movement of glucose across the blood-brain-barrier and the uptake of glucose into neurons and glia (Brown, 2000; B. S. McEwen & Reagan, 2004; Simpson et al., 2007). The crucial role of GLUTs is illustrated by the profound neurological deficits manifested in De Vivo disease, a rare genetic condition in which GLUT subtype 1 is not expressed (De Vivo, Leary, & Wang, 2002). Milder deficits in the expression and translocation of GLUTs have been linked to neuropathology including Alzheimer's

pathology, post-ischemic/hypoxic brain function, and following traumatic brain injury (Cornford, Hyman, Cornford, & Caron, 1996; Hamlin, Cernak, Wixey, & Vink, 2001; Simpson, Chundu, Davies-Hill, Honer, & Davies, 1994; Vannucci, Reinhart, et al., 1998; Vannucci, Seaman, & Vannucci, 1996). Less is known about the range of variation in cerebral GLUT expression in normal conditions and the effects of environmental influences on cerebral GLUT expression.

The GLUT family is not fully characterized, however, current literature indicates at least nine GLUT isoforms are expressed throughout the brain (Brown, 2000; Karim, Adams, & Lalor, 2012), and five of the nine have a plausible role in development (Dwyer & Pardridge, 1993; Garcia-Bueno, Caso, Perez-Nievas, Lorenzo, & Leza, 2007) and a potential role in the effects of chronic stress (Garcia-Bueno et al., 2007; Piroli et al., 2007; Reagan et al., 1999; Reagan et al., 2000). Here we focus on transporter isoforms GLUT 1, 3, 4, 5, and 8. The specific functions of these GLUTs and their involvement in neurologic and stress-related disorders are outlined in **Table 2.1**. GLUT1 and GLUT3 are the primary glucose transporters in the brain, are ubiquitously expressed, and have a high affinity for glucose (Maher, Vannucci, & Simpson, 1994). A strong positive correlation exists between capillary density and GLUT1 in the brain, and a positive correlation also exists between GLUT1 density and local cerebral glucose utilization (Zeller, Rahner-Welsch, & Kuschinsky, 1997). Both GLUT1 and GLUT3 are decreased in the cerebral cortex of Alzheimer's patients (Simpson & Davies, 1994; Zhao et al., 2007) and both have also been implicated in the pathophysiological sequelae that follow both ischemia (Vannucci, Reinhart, et al., 1998) and traumatic brain injury (Bergsneider et al., 1997; Hamlin et al., 2001; Riley & Schneider, 1992). GLUT4, an insulin-responsive glucose transporter, is present in multiple brain regions (Cremerius, 1982) and its translocation in response to insulin is affected by corticosterone (Piroli et al., 2007). GLUT5 is also expressed in the brain but appears to have a low affinity for glucose, and little is known about its cerebral function (Simpson et al., 2007) other than the established ability to transport fructose and localization to microglia (Hartmann et al., 2002). GLUT8 is neuron-specific and is present in the cell bodies of both excitatory and

inhibitory neurons in rat hippocampus (Schouten et al., 1982). Effects of stress on GLUT8 in the hippocampus appear to be dependent on the glycemic state of the organism (Pereira, Hoffman, & Cremer, 1982). Deletion of GLUT8 results in hyperactivity and a reduction in risk assessment behaviors in mice (S. Schmidt et al., 2008) as well as increased neurogenesis without a concomitant change in memory acquisition or retention (Membrez et al., 2006). Few studies have examined multiple cerebral GLUTs in the same animal, and little attention has been given to the influences of sex, age, or stress exposure on the expression of these essential transporters.

Rapid developmental periods, such as puberty, are characterized by both increased cerebral plasticity and augmented demand for metabolic energy. Therefore, changes in GLUT during the adolescent developmental period could lead to longstanding changes in cerebral metabolism and neuron function. Although there is some evidence that stress can modify cerebral GLUT in the adult rat (Garcia-Bueno et al., 2007; Piroli, Grillo, Charron, McEwen, & Reagan, 2004; Reagan et al., 1999), it is unknown whether stress during adolescence can alter cerebral GLUT expression, or whether stress affects GLUT expression similarly in males and females.

Thus, the present study examined the interactions among sex, age, and chronic mixed modality stress on cerebral GLUT mRNA abundance. Because of the dearth of information available regarding cerebral GLUTs during adolescent development and minimal information regarding sex differences, we initially assessed expression of the five GLUT subtypes outlined in four brain regions in male and female adolescent and adult rats. We hypothesized that developmental sex differences in GLUT mRNA abundance would exist in brain regions involved in regulating hormonal secretion and affective behavior, particularly the hypothalamus. In addition, given the substantial energetic demands during adolescence and previous demonstrations of the adverse effects of adolescent stress (Bourke & Neigh, 2011; Bourke et al., 2013), we determined the extent to which chronic stress altered GLUT mRNA abundance in males and females in both adolescence and adulthood. Next, we examined the extent to which

adolescent chronic stress altered GLUT mRNA abundance in the long-term in males and females assessed in adulthood. Finally, given the additional energetic demand posed by an immune challenge, we also examined the extent to which administration of lipopolysaccharide altered GLUT mRNA abundance in adult males and females, a subset of which had undergone chronic adolescent stress, in the most metabolically stress-sensitive region examined, the hippocampus.

Collectively, our data demonstrate that cerebral GLUTs are expressed differentially based on brain region, sex, age, and exposure to stress, either psychosocial or immunologic. Furthermore, the timing of the stress exposure interacts with sex to determine the changes in GLUT mRNA abundance. This study is one of the first reports of multiple cerebral GLUTs with attention to age, sex, environment, and brain region. Regional changes in GLUT expression may impact brain function and thereby behavior.

2.2. Materials and Methods

2.2.1 Animals. Timed pregnant Wistar rats (Charles River, Wilmington, MA) arrived on gestational day 12. This timing of shipping stress is not associated with changes in developmental outcomes (Ogawa, Kuwagata, Hori, & Shioda, 2007b), whereas shipping stress during puberty can have enduring effects on behavior (Laroche, Gasbarro, Herman, & Blaustein, 2009a, 2009b). Rats were housed on a 14:10 reverse light: dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). Rodent diet 5001 chow (Purina Mills, Richmond, IN) and water were maintained *ad libitum* throughout the study. Three days after birth litters were culled and weaned on postnatal day (PND) 23. On PND 36, rats were assigned groups and housed in same-sex pairs. Littermates were assigned to adolescent or adult control or stress groups with no more than two pups per litter assigned to each group. All groups contained between 10 and 12 rats. All animal experiments were approved by Emory University's Institutional Animal Care and Use Committee and carried out in accordance with the National

Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources, 1996). All efforts were made to minimize animal suffering and to reduce the number of rats used.

2.2.2 Experimental Design. For the cohort of rats used to assess regional differences in cerebral GLUT expression as a function of age and sex, rats were maintained in standard colony conditions and adolescent animals were euthanized via rapid decapitation at PND 51 and adult animals were euthanized via rapid decapitation at PND 100. To account for potential variation in circadian or diurnal rhythms in GLUT expression (Corpe & Burant, 1996; Frese, Bazwinsky, Muhlbauer, & Peschke, 2007), all animals were sacrificed between 10am and 12pm, two to four hours before the beginning of their dark cycle. Brains were removed, flash frozen on dry ice, and stored at -80°C until dissection. The hypothalamus, hippocampus, amygdala, and prefrontal cortex were dissected and stored at -80°C until used for quantitative RT-PCR analysis of glucose transporters *Slc2a1*, *Slc2a3*, *Slc2a4*, *Slc2a5*, and *Slc2a8*.

For the assessment of chronic stress effects on cerebral GLUTs, control animals were treated as described above. Rats in the chronic stress cohort were exposed to the chronic mixed modality stress paradigm in either adolescence (PND 37-48) or adulthood (PND 86-97). While adolescence is difficult to define precisely in rats as in humans, it is usually accepted that infancy and early childhood end at weaning and that adulthood begins at PND60. Sex organ maturation occurs roughly from PND35 to PND49, and initiation of sexual behavior as well as additional neurodevelopmental and cognitive changes occur from PND49 to PND60 (McCormick & Mathews, 2007; Spear, 2000). The adolescent stress paradigm thus spans the hormonal surges that occur during the course of development, while the adult paradigm is well past these developmental changes.

For the short-term adolescent and adult cohorts, to ensure that all effects measured were due to the chronic nature of the stress, and not an acute stress, a three-day waiting period was maintained before euthanizing the animals. After rapid decapitation, brains from the adolescent

and adult chronic stress cohorts were treated as described for the control cohort. For the long-term cohort, a four-and-a-half week waiting period (PND48-PND80) was maintained before euthanasia. Stressed rats remained single-housed during this period, while non-stressed rats remained pair-housed. On PND80, this long-term cohort underwent a lipopolysaccharide or saline challenge intraperitoneal lipopolysaccharide (i.p. LPS; E. coli strain 127:B8; 250 µg/kg) or saline injection four hours prior to euthanasia. Rats from this cohort were deeply anesthetized with sodium pentobarbital (1 mg/kg; i.p.). Blood was sampled into heparin-coated syringes via cardiac puncture followed by brief (~1 min) cold saline perfusion to flush circulating macrophages out of the brain. Brains were removed and treated as for the control cohorts.

2.2.3 Mixed Modality Chronic Stress. The mixed modality chronic stress paradigm has previously been described and elicits sex-specific behavioral changes in adolescent rats (Bourke & Neigh, 2011; Bourke et al., 2013; Pyter, Kelly, Harrell, & Neigh, 2013). Briefly, adolescent and adult stress was defined as individual housing beginning at PND37 (adolescents) or PND 86 (adults) and continuing throughout the study combined with randomly alternating daily exposure to either 6 social defeat episodes and 6 restraint sessions (PND 37-48 adolescent cohort and PND 86-97 adult cohort; 6 days each of social defeat and restraint). Social defeat occurred by pairing the rats with a same sex aggressive Long Evans rat (female aggressors were ovariectomized). Pairing lasted for 30 minutes on each social defeat day. Rats interacted for 3 pins or 5 minutes and were then separated by a screen for the remainder of the 30 minutes. The control groups remained pair-housed with a same sex littermate throughout the study. The study was not designed to assess the specific effects of individual housing, restraint, or social defeat, but was designed to use this combination of established stressors to induce chronic stress during adolescence or adulthood. Body mass was monitored throughout the study for all groups.

2.2.4 Vaginal Lavage. Beginning at PND94, vaginal lavage was performed on adult females to determine the estrous cycle stage at time of death. Cycle was characterized as diestrus 1 (D1), diestrus 2 (D2), pro-estrus (P) or estrus (E) based on methods previously described (J. M.

Goldman, Murr, & Cooper, 2007; Westwood, 2008). There were no significant differences in distribution of animals in a given cycle stage among the different experimental cohorts, therefore, reported group effects are not the result of a confounding effect of estrous cycle stage. However, this study design does not rule out the possibility of effects of estrous cycle stage on mRNA abundance of GLUTs.

2.2.5 Quantitative RT-PCR. RNA was extracted from frozen rat tissue consisting of hypothalamus, hippocampus, amygdala, and prefrontal cortex using the mirVana PARIS KIT (Ambion Life Technologies, Carlsbad, CA). RNA quantity and integrity was assessed using a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). RNA was then standardized so all samples started reverse transcription with 1ug RNA. Then, RNA was reverse-transcribed using High Capacity RNA to cDNA Kit (Applied Biosystems, Foster City, CA). Several rat endogenous control plates (*Tfrc*, *Hprt1*, *Ubc*, and *Ppia*; Applied Biosystems, Foster City, CA) were run in order to determine the optimal endogenous control, but none of the endogenous controls nor their geometric means tested remained invariant among all experimental groups. Thus, to ensure standardization in total cDNA across groups, cDNA was quantified via the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized so that all samples started quantitative RT-PCR with 1ug cDNA. TaqMan gene expression master mix and rat TaqMan Gene Expression Assays were purchased from Applied Biosystems (Carlsbad, CA) with probes labeled for *Slc2a1* (GLUT1; assay ID Rn01417099_m1), *Slc2a3* (GLUT3; assay ID Rn00567331_m1), *Slc2a4* (GLUT4, assay ID Rn01752377_m1), *Slc2a5* (GLUT5; assay ID Rn00582000_m1), and *Slc2a8* (GLUT8; assay ID Rn00585203_m1). The universal two-step RT-PCR cycling conditions used on the 7900HT Sequence Detection System (Applied Biosystems) were: 50^{C} (2 min), 95^{C} (10 min), 40 cycles of 95^{C} (15 s) and 60^{C} (1 min). Relative gene expression of individual samples were run in triplicate and calculated by the comparative Ct quantification method relative to the same-sex no stress saline control group or the male adolescent no stress saline group ($2^{-\Delta CT}$) with a Grubb's correction for statistical outliers. The

coefficient of variance cut off was set to 4%. All TaqMan gene expression assays are guaranteed to have 90 - 100% amplification efficiency as determined by the genome-aided probe and primer design pipeline and reported in the "Amplification Efficiency of TaqMan Gene Expression Assays" Application Note 127AP05-03 from Life Technologies.

2.2.6 Statistical Analysis. To determine the effects of sex and developmental stage on GLUT gene expression, statistical comparisons of differences in RT-PCR for the hypothalamus, hippocampus, amygdala, and prefrontal cortex were analyzed using Two-Way ANOVA followed by Tukey's post-hoc testing, with all groups normalized to adolescent male values. Percent change in body mass before and after stress was also analyzed by two-way ANOVA with the factors of sex and stress. The effect of chronic stress on weight gain was separately assessed in adolescence and adulthood by two-way ANOVAs due to the large disparity in adolescent versus adult weight at baseline. To determine the effects of stress and developmental stage on GLUT gene expression, all groups were analyzed within their given sex (male or female) and normalized to the adolescent control group. Two-way ANOVA was performed using the factors of stress and developmental stage followed by Tukey's post-hoc testing. All statistical analysis was performed using GraphPad Prism v.6 (La Jolla, CA). All data were determined to be statistically significant when $p < 0.05$ and are presented as a mean ± standard error of the mean (SEM).

2.3. Results

2.3.1 Hypothalamic GLUT1 and GLUT4 mRNA Abundance Increases with Age and is Sexually Dimorphic Hypothalamic expression of *Slc2a1*as measured using RT-PCR increased with age for male, but not female rats (interaction of sex and age: $F_{1, 34} = 4.286$; $p = 0.046$; main effect age: $F_{1, 34} = 4.395$; $p = 0.043$; **Figure 2.1a**). A sex difference in hypothalamic *Slc2a1* expression also existed (main effect sex: $F_{1, 34} = 4.392$; $p = 0.043$) and was specific to lower expression of hypothalamic *Slc2a1* expression in male than female adolescent rats ($p = 0.028$). A

similar sex and age dependent pattern of gene expression was observed for *Slc2a4* in the hypothalamus such that *Slc2a4* expression increased with age for male but not female rats (interaction of sex and age: $F_{1, 34} = 6.332$; $p = 0.0167$; main effect of sex: $F_{1, 34} = 4.787$; $p = 0.035$; **Figure 2.1b**). mRNA transcripts that encode for GLUT isoforms 3 (sex: $F_{1,32} = 2.968$; $p = 0.0946$; age: $F_{1,32} = .067$; $p = 0.796$), 5 (sex: $F_{1,34} = 0.955$; $p = 0.335$; age: $F_{1,34} = 0.163$; $p = 0.688$), and 8 (sex: $F_{1,33} = 2.767$; $p = 0.105$; age: $F_{1,33} = 1.121$; $p = 0.297$) in the hypothalamus did not differ based on either sex or age.

2.3.2 Hippocampal mRNA Abundance of GLUT 3, 4, and 5 Decreases with Age in Males and Females Hippocampal expression of *Slc2a3, Slc2a4,* and *Slc2a8* was reduced in male and female adults (main effects of age: *Slc2a3*: $F_{1,35} = 4.211$, $p = 0.047$, **Figure 2.2a**; *Slc2a4*: $F_{1,35} = 4.328$, $p = 0.044$, **Figure 2.2b**; and *Slc2a8*: $F_{1,34} = 5.415$, $p = 0.026$, **Figure 2.2c**). No effect of sex or age on expression of Slc2a1 (sex: $F_{1,35} = 0.030$; $p = 0.861$; age: $F_{1,35} = 0.899$; $p = 0.349$) or *Slc2a5* (sex: $F_{1,36} = 0.0008$; $p = 0.977$; age: $F_{1,36} = 2.070$; $p = 0.158$) was observed in the hippocampus.

2.3.3 Neither Sex nor Age Impact mRNA Abundance of GLUTs in the Amygdala or Prefrontal Cortex Unlike the hypothalamus and hippocampus, expression of genes encoding for GLUT 1, 3, 4, 5, and 8 did not differ between adolescent and adult rats ($p > 0.05$) or between male and female rats ($p > 0.05$) within the amygdala or the prefrontal cortex.

2.3.4 Chronic Stress Reduces Weight Gain Exposure to chronic stress during adolescence reduced the percent weight gained (main effect of stress: $F_{1,39} = 204.8$; $p<0.001$) and preferentially reduced male weight gain (main effect of sex $F_{1,39} = 21.34$; $p < 0.001$; interaction of sex and stress $F_{1,39} = 6.608$; $p = 0.014$**;** mean percent change ± standard deviation: adolescent male control: 49.29% ± 4.22%; adolescent male stress: 40.38% ± 4.61%; adolescent female control: 28.37% ± 4.11%; adolescent female stress: 25.834 ± 3.23%). Among the adult rats, exposure to chronic stress reduced percent weight gain in both males and females (main effect of stress: $F_{1,38} = 15.57$; $p < 0. 001$) without a significant sex effect ($F_{1,38} = 0.008$; $p=0.927$; mean

percent change ± standard deviation: adult male control: 4.10% ± 3.31%; adult male stress: 1.22% ± 2.24%; adult female control: 4.79% ± 2.16%; adult female stress: 0.37% ± 3.74%). Prior to stress, body masses for each cohort were (in grams; mean ± standard deviation): male adolescent control, 178.7 ± 14.2, male adolescent pre-stress, 177.8; female adolescent control, 143.7 ± 8.3; female adolescent pre-stress, 143.2 ± 8.8; male adult control, 456.3 ± 52.6; male adult pre-stress, 459.7 ± 38.1; female adult control, 257.7 ± 18.4; and female adult pre-stress, 258.8 ± 10.7. Post-stress, body masses for each cohort were (in grams; mean ± standard deviation): male adolescent control, 266.5 ± 18.9; male adolescent post-stress, 249.5 ± 15.1, female adolescent control, 184.6 ± 14. 3, female adolescent post-stress, 180.1 ± 10.9; male adult control, 475.4 ± 60.1; male adult post-stress, 465 ± 41.2; female adult control, 270.1 ± 20.6; and female adult post-stress, 259.7 ± 12.0.

2.3.5 Chronic Stress Interacts with Stress and Age to Alter Hypothalamic GLUT mRNA Abundance Hypothalamic expression of *Scl2a1* (**Figure 2.3a** and **Figure 2.3b**) and *Scl2a8* (**Figure 2.3g** and **Figure 2.3h)** was not altered after chronic stress in either males or females at either age assessed ($p > 0.05$). In contrast, hypothalamic expression of *Slc2a3* decreased after chronic stress for female, but not male rats (main effect of stress: $F_{1,37} = 5.67$; $p = 0.022$; **Figure 2.3d**). While *Slc2a4* expression remained unchanged among females (stress: $F_{1,39} = 1.910$; $p = 0.174$; age: $F_{1,39} = 0.101$; $p = 0.751$), chronic stress and age interacted to affect *Slc2a4* gene expression in males such that stress increased expression in adolescent males and decreased expression in adult males (interaction of stress and age: $F_{1,33} = 6.980$; $p = 0.012$; **Figure 2.3e**). However, consistent with the data presented in Figure 1b, post-hoc testing revealed significant increases in *Slc2a4* expression in male adult controls relative to male adolescent controls ($p = 0.048$) indicating that this effect may be due to differential baseline expression across age.

2.3.6 Chronic Stress Interacts with Stress and Age to Increase Hippocampal GLUT mRNA Abundance Male hippocampal *Slc2a1* expression increased after exposure to chronic

stress, irrespective of age (main effect of stress: $F_{1,33} = 4.9$; $p = 0.033$; **Figure 2.4a**). Expression of *Slc2a1* was the only gene assessed that was altered by chronic stress in the male hippocampus. Female expression of hippocampal *Slc2a3* decreased in adulthood (main effect of age: $F_{1,39} = 4.871$; $p = 0.033$; **Figure 2.4d**), but exposure to chronic stress altered this pattern to increase *Slc2a3* expression in adulthood, nullifying the age-related reduction in gene expression. Chronic stress also increased expression of *Slc2a5* in the female hippocampus irrespective of age (main effect of stress: $F_{1,40} = 4.06$; $p=0.036$; **Figure 2.4f**). Female expression of hippocampal *Slc2a8* showed an overall decrease with age ($F_{1,34}=5.415$; $p=0.036$; **Figure 2.4h**) and as with *Slc2a3*, chronic stress altered this pattern to increase *Slc2a8* expression in adulthood, nullifying the age-related reduction in gene expression. Hippocampal expression of *Slc2a4* was not altered by stress in either males ($F_{1,32}=0.150$; $p=0.700$) or females ($F_{1,40}=0.003$; $p=0.955$) at any age.

2.3.7 Chronic Stress Interacts with Stress and Age to Increase GLUT mRNA Abundance in the Male Amygdala In the male amygdala, the increase in *Slc2a3* expression with age (main effect of age: $F_{1,37} = 5.07$; $p = 0.030$; **Figure 2.5c**) was further exacerbated by an increase in *Slc2a3* expression after chronic adult stress, as determined in post-hoc analysis ($p= 0.045$). Chronic stress also interacted with age to alter amygdalar expression of *Slc2a5* (interaction of age and stress: $F_{1,33}=6.601$; $p=0.014$; **Figure 2.5e**) such that expression increased with chronic stress exposure in adolescent males, but decreased with chronic stress exposure in adult males. Amygdalar expression of *Slc2a1* ($F_{1,34}=1.053$; $p=0.312$), *Slc2a4* ($F_{1,37}=1.572$; $p=0.217$), and *Slc2a8* ($F_{1,32}=0.059$; $p=0.808$) was unaltered by chronic stress in males, regardless of age. Likewise, no GLUT gene isoforms were altered by stress in the female rat amygdala, regardless of age: *Slc2a1* ($F_{1,38}=1.297$; $p=0.261$), *Slc2a3* ($F_{1,39}=1.635$; $p=0.208$), *Slc2a4* ($F_{1,37}=1.572$; $p=0.217$), *Slc2a5* ($F_{1,37}=0.013$; $p=0.909$), *Slc2a8* ($F_{1,36}=0.348$; $p=0.558$).

2.3.8 *Chronic Stress Does Not Alter GLUT mRNA Abundance in the Prefrontal Cortex.* In the prefrontal cortex, gene expression of *Slc2a1*, *Slc2a3*, *Slc2a4*, *Slc2a5*, and *Slc2a8* were also analyzed using the same parameters as for the other brain regions. No significant

effects of stress, age, or sex were found for any GLUTs analyzed in the prefrontal cortex ($p > 0.05$ in all cases).

2.3.9 Effects of Adolescent Chronic Stress on Hippocampal GLUT mRNA Abundance Persist into Adulthood in a Sexually Dimorphic Manner At PND80, four and a half weeks after the end of chronic adolescent stress, sex and stress significantly interacted to affect *Slc2a1* expression in the hippocampus (**Figure 2.6a,** $F_{1,35}=11.95$; $p=0.002$). Though neither sex ($F_{1,35}=0.1965$; $p=0.660$) nor stress ($F_{1,35}=3.125$; $p=0.086$) independently altered *Slc2a1* expression, post-hoc testing indicated that control female rats had significantly higher *Slc2a1* expression than control males ($p=0.047$), while stressed female rats had significantly lower *Slc2a1* expression than control males ($p=0.004$). Sex significantly increased hippocampal *Slc2a4* expression (**Figure 2.6b,** $F_{1,34}=11.72$; $p=0.002$) in this cohort irrespective of stress history ($F_{1,34}=0.326$; $p=0.572$). Post-hoc testing, however, showed that this effect was due to increased relative expression of *Slc2a4* among stressed and control females vs. stressed males. No long-term effect of stress or sex effect was observed for *Slc2a3* (stress: $F_{1,35}=1.007$; $p=0.323$; sex: $F_{1,35}=0.066$; $p=0.799$), *Slc2a5* (stress: $F_{1,35}=1.273$; $p=0.267$; sex: $F_{1,35}=1.244$; $p=0.272$), and *Slc2a8* (stress: $F_{1,35}=2.355$ $p=0.134$; sex: $F_{1,35}=2.153$; $p=0.151$).

2.3.10 Adolescent Chronic Stress Blunts the Male Increase in Hippocampal GLUT mRNA Abundance after LPS Male rats generally showed greater changes in hippocampal GLUT mRNA abundance in response to LPS injection at PND80 than female rats (**Figure 2.8**). LPS both independently influenced *Slc2a1* expression (**Figure 2.7a**; $F_{1,35}=5.265$; $p=0.028$) and interacted with stress to alter expression ($F_{1,35}=6.915$; $p=0.013$) in males. Post-hoc testing demonstrated that LPS injection only elevated *Slc2a1* expression non-stressed males, but not stressed males ($p=0.008$). Similarly, both LPS (**Figure 2.7e;** $F_{1,36}=37.25$; $p<0.0001$) and prior stress ($F_{1,36}=20.98$; $p<0.0001$) independently affected *Slc2a5* expression in males, and LPS and prior stress also interacted ($F_{1,36}=5.429$; $p=0.026$) to influence male *Slc2a*5 hippocampal expression. Post-hoc testing again demonstrated that LPS injection only elevated *Slc2a5*

expression non-stressed males, but not stressed males ($p<0.0001$). LPS additionally altered *Slc2a8* expression in males (**Figure 2.7g;** $F_{1,36}=4.920$; $p=0.033$) without a main effect of stress ($F_{1,36}=0.440$; $p=0.511$). However, post-hoc testing once more showed that LPS injection only elevated *Slc2a8* expression non-stressed males, but not stressed males ($p=0.034$). Neither LPS nor prior history of stress influenced *Slc2a3* (LPS: $F_{1,36}=0.029$; $p=0.867$; stress: $F_{1,36}=0.207$; $p=0.652$) or *Slc2a4* expression (LPS: $F_{1,36}=1.476$; $p=0.232$; stress: $F_{1,36}=4.004$; $p=0.053$), though there was a compelling trend toward an effect of adolescent stress to reduced hippocampal *Slc2a4* expression in adult males.

In females, only hippocampal *Slc2a1* expression was influenced by either LPS or prior stress history **(Figure 2.7b).** Both LPS ($F_{1,36}=6.718$; $p=0.014$) and a prior history of stress ($F_{1,36}=6.585$; $p=0.015$) altered *Slc2a1* expression in females, and LPS and stress additionally interacted ($F_{1,36}=4.539$; $p=0.040$) to influence *Slc2a1* expression. Post-hoc testing indicated that these effects were due to a significant decrease in *Slc2a1* expression in saline-treated stressed females relative to saline-treated non-stressed females ($p=0.011$), but that LPS-treated stressed or non-stressed females did not differ in *Slc2a1* expression. There was a potential trend for LPS to increase *Slc2a5* expression in females ($F_{1,35}=2.998$; $p=0.092$), but *Slc2a5* expression was affected neither by prior stress ($F_{1,36}=0.3544$; $p=0.555$) nor an interaction between LPS and prior stress ($F_{1,36}=702$; $p=0.408$). Neither LPS nor a prior history of stress influenced hippocampal *Slc2a3, Slc2a4,* or *Slc2a8* expression in females (all $p>0.10$).

2.4. Discussion

Sex and age modify regional and isoform-specific GLUT expression in the brain. Sex differences in GLUT mRNA abundance were most profound in the hypothalamus, while the transition from adolescence to adulthood appeared to be most influential on GLUT transcript expression in the hippocampus. Exposure to chronic stress impacted GLUT expression

differentially depending on age and sex, and effects of chronic stress on GLUT expression were evident in the hypothalamus, hippocampus, and amygdala – but not in the prefrontal cortex. Adolescent stress had a long-term and sexually dimorphic effect on hippocampal GLUT expression, lasting into adulthood up to four and a half weeks after the end of the stress period. Finally, inflammatory stress in the form of LPS primarily increased adult male hippocampal GLUT expression, an effect that was blunted by a prior history of adolescent stress. In summary, GLUT mRNA abundance is plastic in the adolescent and adult brain and plasticity in GLUT isoform expression differs by brain region. Furthermore, sex is an important variable in assessing the impact of stress, both psychosocial and physiological, on GLUT transcript expression. Given the critical role of GLUT in supplying energy to the brain, understanding the effects of sex, development, and stress on GLUT expression is an essential first step in developing a comprehensive knowledge regarding the potential role of pathological changes in GLUTs in neuronal and behavioral dysfunctions.

In the hypothalamus, *Slc2a1* and *Slc2a4* expression increased with age in males (**Figure 2.1**). Female adolescent rats had higher expression of *Slc2a1* and *Slc2a4* than adolescent males and no effect of age was observed in the females. The increase in male hypothalamic *Slc2a1* expression is consistent with developmental patterns of GLUT1 identified in the hypothalamus (Vannucci, 1994). The fact that the female hypothalamus did not undergo similar increases in *Slc2a1* may be due to sex differences in developmental timing, given the potential for females to undergo puberty earlier in life (Sisk & Foster, 2004). Moreover, significant sexual dimorphism exists in the hypothalamus (Gorski, Gordon, Shryne, & Southam, 1978; Swaab, Chung, Kruijver, Hofman, & Ishunina, 2002), and others have reported similar divergence in the onset at which males and females reach adult levels of other hypothalamic neuropeptides (Clarkson & Herbison, 2006).

Expression of GLUTs in the hippocampus was more sensitive to the effects of the adolescent to adult transition than to sex differences (**Figure 2.2**). Hippocampal expression of

Slc2a3, Slc2a4, and Slc2a8 decreased in adulthood in both sexes. This decrease in mRNA abundance is consistent with the peak and subsequent decrease in GLUT4 and GLUT8 protein seen in mouse whole brain homogenate between PND21 and PND60 (Sankar, Thamotharan, Shin, Moley, & Devaskar, 2002). To understand this developmental shift, it is crucial to understand the cellular and regional distribution of these transporters. GLUT4 (El Messari, Ait-Ikhlef, Ambroise, Penicaud, & Arluison, 2002) and GLUT8 (Ibberson et al., 2002) have previously been shown to be enriched in the hippocampus. GLUT3, GLUT4, and GLUT8 are primarily expressed in neurons (B. S. McEwen & Reagan, 2004), with GLUT4 primarily dendritic and GLUT8 localized in both the soma and neuronal processes (Reagan et al., 2002). This localization and redundancy is suggestive of a role in neurotransmission. During puberty, the hippocampus undergoes significant dendritic pruning and reorganization(Meyer, Ferres-Torres, & Mas, 1978; Yildirim et al., 2008) that plays a critical role in regulating cognitive and affective behavior in adulthood (Sisk & Foster, 2004; Sisk & Zehr, 2005). Reductions in hippocampal neuronal GLUTs may reflect this pruning and represent an overall shift toward an adult dependence on neuronal-glial interactions for nutrient transport.

The shift to increased hypothalamic GLUT1 gene expression with age, but the decrease in hippocampal expression of GLUT3, GLUT4, and GLUT8 is additionally intriguing in light of the developmental shift from the use of ketones to glucose as a metabolic substrate (Nehlig, 2004). Throughout the suckling phase, a high level of β-hydroxybutyrate as well as monocarboxylate transporters, found in endothelial cells and astrocytes, are maintained to allow for metabolism and transport of ketone bodies due to the high fat content of milk (Nehlig, 2004). Given GLUT1's expression on similar cell types to monocarboxylate transporters, it is not surprising that the data reflect an age-dependent increase in GLUT1 expression coincident with the known developmental transition to preferentially transport glucose over ketone bodies and alternate substrates such as pyruvate and lactate. The other glucose transporters shown here to decrease in expression with age have different cell type expression (B. S. McEwen & Reagan,

2004), have lower affinity for glucose (Brown, 2000), and have further not been shown to play the same critical role in glucose sensing as hypothalamic GLUT1(Chari et al., 2011).

We next assessed the effects of chronic mixed modality stress on regional GLUT expression in male and female adult and adolescent rats to determine whether chronic stress would alter cerebral GLUT expression. The data presented here show that chronic stress exposure modulates GLUT mRNA abundance in a sexually dimorphic manner in the hypothalamus (**Figure 2.3**), hippocampus (**Figure 2.4**), and amygdala (**Figure 2.5**), but not in the prefrontal cortex. Chronic stress significantly altered cerebral GLUT transcription in males and females throughout both developmental stages but in a sexually dimorphic and region-specific manner. Broadly speaking, in the hypothalamus, chronic stress altered expression of specific transporters in a sexually dimorphic manner; in the hippocampus, chronic stress increased expression of specific transporters in both males and females; and in the amygdala, stress affected only male transporter expression.

Consistent with previous data indicating a male bias to the metabolic effects of stress (Bourke & Neigh, 2011; Pyter et al., 2013), male glucose transporter expression increased in most cases after chronic stress: hypothalamic *Slc2a4* increased after adolescent stress; hippocampal *Slc2a1* increased after stress at both ages; and amygdalar *Slc2a3* and *Slc2a5* after adult and adolescent stress, respectively. Collectively, these data suggest an overall increase in cerebral glucose requirement after chronic stress, similar to the increase in glucose demand and GLUT1 expression in states of physiological stress including hypoxia (Boado & Pardridge, 2002), immune cell activation (Maratou et al., 2007), or vascular injury (Adhikari et al., 2011). Regional differences in isoform-specific regulation in response to stress may be due to relative baseline expression of the transporters in each region as well as each transporter's specific role. For example, regional cerebral blood flow is tightly linked to energy metabolism (Koehler, Roman, & Harder, 2009; Sokoloff, 1989) and highly vascularized regions may be more susceptible to changes in expression of the blood-brain-barrier and astrocyte transporter GLUT1. The

hypothalamus is a region of both critical hormonal and metabolic control (Williams et al., 2000) and may play an important role in glucose and insulin-sensing (Marty, Dallaporta, & Thorens, 2007); thus, GLUT4 may be particularly susceptible to stress influence in this region. Peripherally, GLUT4 has been shown to be upregulated in response to prenatal stress in male, but not female, placenta (Mueller & Bale, 2008). In addition, other GLUT isoforms may be affected by stress paradigms under specific metabolic constraints based on each isoform's functional role. For example, others have shown that male hippocampal *Slc2a8* levels are relatively unchanged when exposed to short-term restraint stress (Piroli et al., 2004), consistent with the male hippocampal *Slc2a8* expression data shown here. However, streptozotocin-induced diabetes dramatically increases hippocampal *Slc2a8* expression (Piroli et al., 2004), yet this affect is significantly attenuated when exposed to the short-term restraint stress (Piroli et al., 2004). Thus, stress effects on GLUT isoforms can be environmentally dependent, as demonstrated in our results by the sexual dimorphism in stress-induced GLUT gene expression.

To explore the long-term effects of chronic stress on cerebral GLUT expression and the interaction of psychosocial stress with an inflammatory stressor, male and female adolescents that underwent chronic mixed modality stress or their control counterparts were housed until PND80. At PND80, animals were submitted to either an LPS or saline challenge and hippocampal tissue was examined. At baseline, the long-term effects of stress were confined to *Slc2a1* and *Slc2a4*, and indicated sexual dimorphism (**Figure 2.6**). Females stressed as adolescents reduced expression of *Slc2a1*, while stressed males had a lower expression of *Slc2a4* relative to both control and non-stressed females. The long-term effects of stress on cerebral GLUT expression became much more pronounced, however, when considered in the context of the LPS challenge. LPS upregulated hippocampal *Slc2a1, Slc2a5,* and *Slc2a8* in non-stressed adult males **(Figure 2.7).** However, stressed males did not upregulate any of these GLUT isoforms. Moreover, neither stressed nor non-stressed females altered hippocampal GLUT expression in response to

LPS. Indeed, the only effect in females in this cohort was a reduction in *Slc2a1* expression in saline-treated stressed animals relative to their non-stressed counterparts.

Inflammation has a profound effect on glucose metabolism. During moderate to severe infection, the glucose production rate may increase 150-200%, with correspondingly high glucose levels . This hyperglycemia is partially due to increases in hepatic gluconeogenesis associated with a glucocorticoid-mediated stress response, and glucose uptake is diverted from skeletal tissue and "non-essential" functions to support critical organs (Michie, 1996). When chronically administered, LPS can even induce insulin resistance and promote increased adipose storage (Cani et al., 2007). Given this literature, it is thus unsurprising that hippocampal GLUT expression increased in males. What is more surprising, perhaps, is the lack of response in females and the impaired response to LPS in the context of adolescent stress. A complex relationship exists between neuroinflammation and stress, with stress transiently increasing pro-inflammatory cytokine expression in limbic brain regions (Nguyen et al., 1998). Longer term effects of stress are sometimes undetectable without an additional challenge, such as the LPS challenge used here (Frank, Baratta, Sprunger, Watkins, & Maier, 2007; Gibb, Hayley, Poulter, & Anisman, 2011; Girotti, Donegan, & Morilak, 2011; J. D. Johnson, O'Connor, Hansen, Watkins, & Maier, 2003). Work from our lab examining the effects of LPS after chronic adolescent stress has additionally found a similar sexual dimorpism in the response, with males uniquely susceptible (Pyter et al., 2013). It is possible that sex steroid may mediate some of this sexual dimorphism, as Pyter et al observed that the blunted response to LPS in stressed females coincided with an increase in plasma estradiol. Estradiol has been shown to reduce microglial-derived inflammatory markers *in vitro* (Bruce-Keller et al., 2000; Dimayuga et al., 2005; Smith, Das, Butler, Ray, & Banik, 2011; Tapia-Gonzalez, Carrero, Pernia, Garcia-Segura, & Diz-Chaves, 2008). However, others have demonstrated that estradiol can potentiate an inflammatory response in microglia (Loram et al., 2012; Soucy, Boivin, Labrie, & Rivest, 2005) and further work will be necessary to understand the sexually dimorphic cerebral metabolic response to LPS.

Though chronic psychosocial stress and inflammatory stress induced primarily upregulatory effects on GLUT mRNA abundance in males, it did not have such a consistent effect in females. Chronic stress reduced *Slc2a3* expression in the hypothalamus in both adult and adolescent females. Stress is known to alter the HPG axis, to the extent of causing irregular estrous cycles in female rats (Gonzalez, Rodriguez Echandia, Cabrera, & Foscolo, 1994), and this reduction in the primary neuronal transporter GLUT3 in the hypothalamus in females may be part of this interaction between the HPA and HPG axes. To control for cycle stage, females were examined using vaginal cytology for several days prior to tissue collection to permit for a balance of cycle stage at endpoint collection in both the stressed and control groups. Although the current study does not address the question of whether or not GLUT expression varies across the estrous cycle, the design used does rule out the possibility that the reported effects in females are due to unequal representation of estrous cycle stages among the groups.

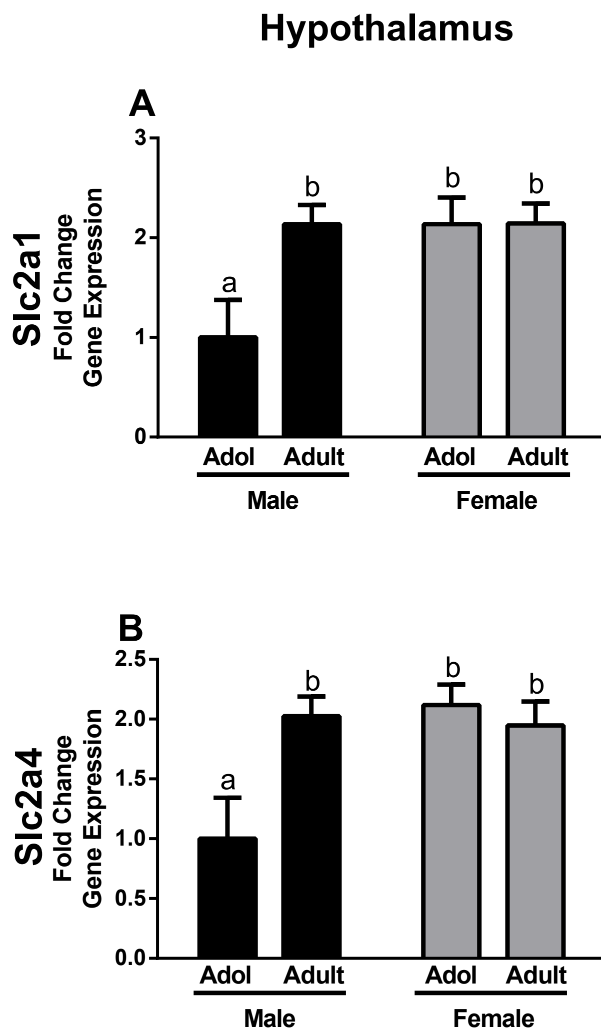
Collectively, the data presented establish that sex, age, and stress all impact gene expression of GLUTs in an isoform- and brain region- specific manner. The data demonstrate global increases in hypothalamic GLUT mRNA abundance in adulthood in males, while female hypothalamic GLUT expression is less plastic from adolescence to adulthood. Consistent with the literature on hippocampal reorganization, we demonstrate decreases in hippocampal transporter expression from the adolescent to adult transition, regardless of sex. Chronic stress effects are complex and affect expression profiles in region- and isoform-specific ways. Stress robustly increased male hippocampal *Slc2a1* expression and altered male amygdala expression, while females showed more regional variability between the hypothalamus and hippocampus in response to stress with both increased and decreased expression depending on the isoform. If these differences in mRNA abundance translate into differences in GLUT protein and function, then these variations may represent alternate energetic strategies that could underlie sex-specific differences in developmental and neurological outcomes and susceptibilities to stress-induced alterations in physiology and behavior.

Table 2.1.

Protein	Gene	Cerebral Location	Involvement in Neurologic Disease and the Stress Response	References
GLUT1	*Slc2a1*	Astrocytes; neurons Endothelial cells	Astrocyte transporter (45 kDa); Blood brain barrier transporter (55 kDa) Deficiency in De Vivo's Disease Decreased expression in Alzheimer's Disease, traumatic brain injury, ischemia	(Bergsneider et al., 1997; De Vivo et al., 2002; Hamlin et al., 2001; Leino, Gerhart, van Bueren, McCall, & Drewes, 1997; Morgello, Uson, Schwartz, & Haber, 1995; Simpson, Vannucci, & Maher, 1994)
GLUT3	*Slc2a3*	Neurons; neuropil	Decreased expression in Alzheimer's Disease, traumatic brain injury, ischemia Reduced expression in rat forebrain after chronic stress exposure	(Bergsneider et al., 1997; Hamlin et al., 2001; Simpson, Chundu, et al., 1994; Vannucci, Reinhart, et al., 1998)
GLUT4	*Slc2a4*	Neurons; somatodendritic	Insulin sensitive glucose transporter Impaired insulin-induced translocation after corticosterone treatment in rat hippocampus	(Choeiri, Staines, Miki, Seino, & Messier, 2005; B. S. McEwen & Reagan, 2004; Piroli et al., 2007; Simpson et al., 2007)
GLUT5	*Slc2a5*	Microglia	Microglial localization; involvement in inflammatory response	(Malide, Davies-Hill, Levine, & Simpson, 1998; B. S. McEwen & Reagan, 2004)
GLUT8	*Slc2a8*	Neurons; somatodendritic	Depletion results in increased neurogenesis, hyperactivity, and reduction in risk assessment behaviors without memory alteration in mice	(Membrez et al., 2006; Reagan et al., 2002; S. Schmidt, Joost, & Schurmann, 2009)

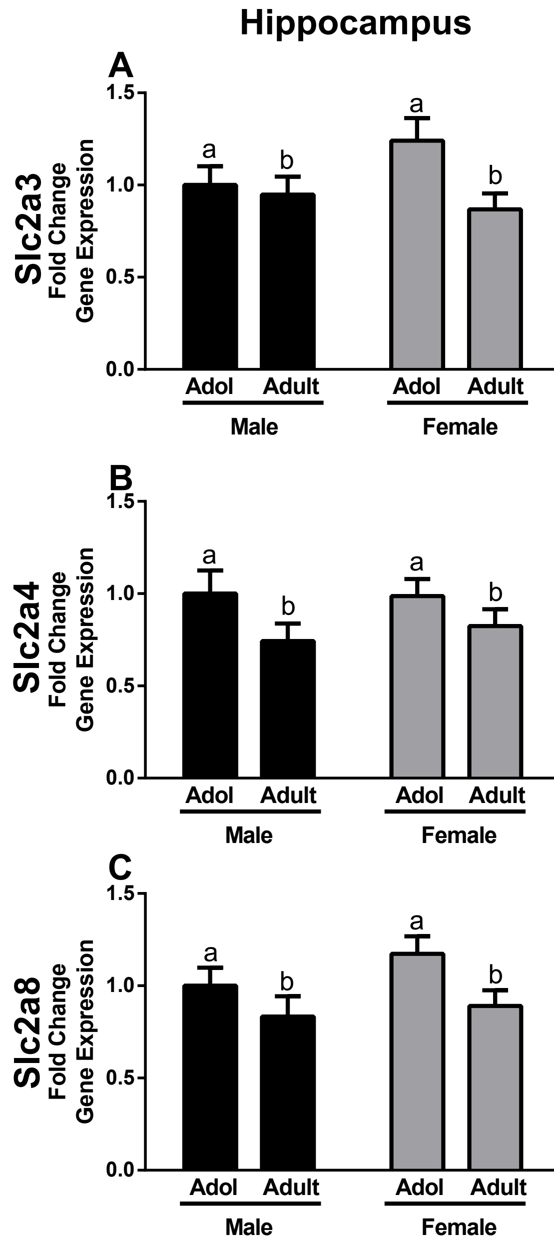
This table summarizes the primary cerebral glucose transporters potentially involved in neurological disease or stress responses.

Figure 2.1.



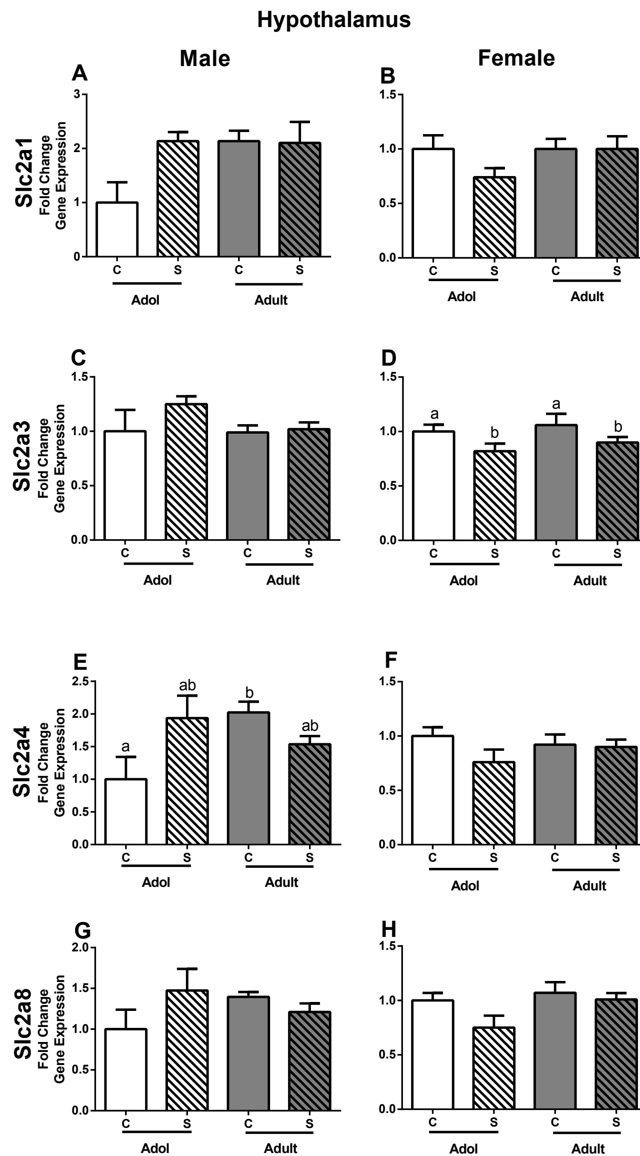
Adolescent male rats express less GLUT1 and GLUT4 in the hypothalamus than either adult males or females. (A) GLUT1 (*Slc2a1)* expression increased with age. In addition, male adolescents had lower expression relative to female adolescents. (B) GLUT4 (*Slc2a4)* gene expression also increased with age in males. Similar to *Slc2a1,* a sex difference existed in *Slc2a4* expression. Data shown represent mean fold change $2^{-\Delta CT}$ ± SEM. Different letters indicate significant differences in post-hoc testing with $p<0.05$.

Figure 2.2.



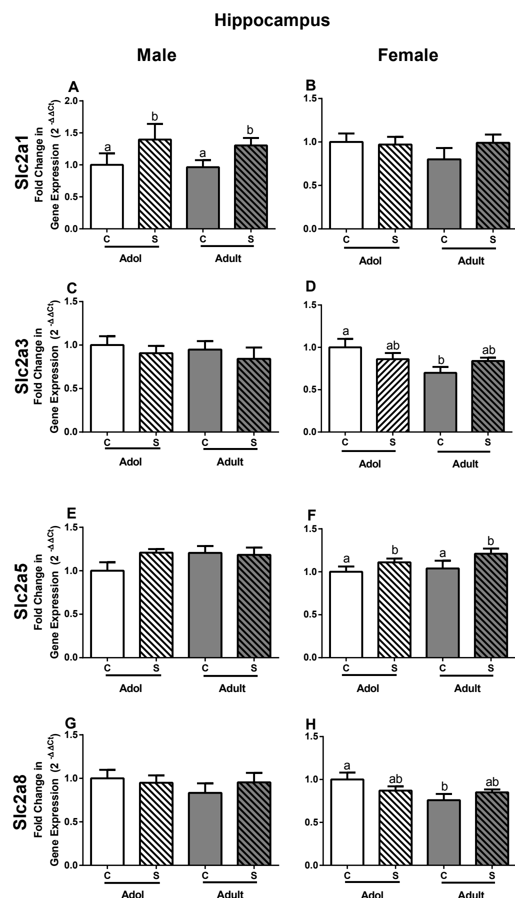
Age determines gene expression of GLUT3, GLUT4, and GLUT8 in the hippocampus, regardless of sex. Male and female adults had reduced hippocampal expression compared to adolescents for (A) GLUT3 (*Slc2a3*, (B) GLUT4 (*Slc2a4)*, and (C) GLUT8 (*Slc2a8)*. Data shown represent mean fold change $2^{-\Delta CT}$ ± SEM. Different letters indicate significant differences in post-hoc testing with $p<0.05$.

Figure 2.3.



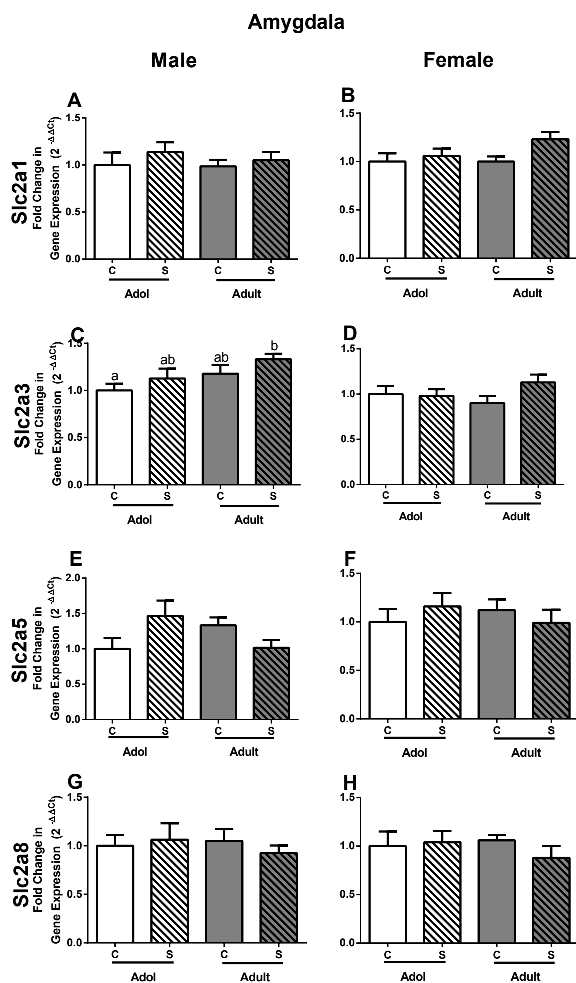
Stress differentially alters GLUT expression in the hypothalamus of male and female rats. (D) GLUT3 (*Slc2a3)* gene expression decreased in adolescent and adult females when exposed to stress regardless of age. (E) GLUT4 (*Slc2a4)* expression in males increased with stress in adolescents and decreased with stress in adults. Data shown represent mean fold change $2^{-\Delta CT}$ ± SEM. Different letters indicate significant differences in post-hoc testing with $p<0.05$. C=Control; S=Stress.

Figure 2.4.



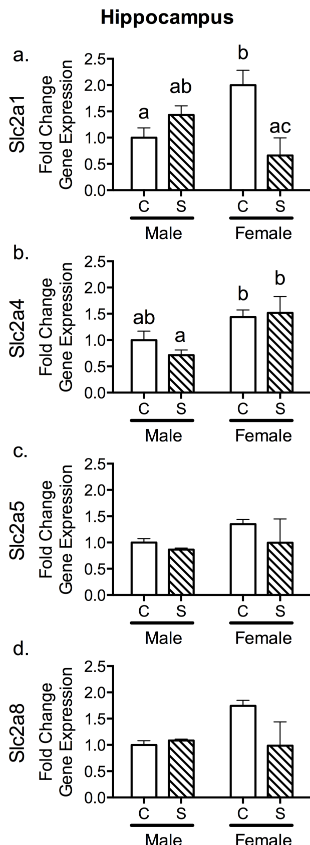
Chronic stress, age, and sex interact to dictate GLUT expression in the hippocampus. (A, B) Chronic stress increased expression of GLUT1 (*Slc2a1)* in the hippocampus of male but not female rats. (C, D) GLUT3 (*Slc2a3)* decreased in female adult controls but was unaffected in males. (E,F) GLUT5 (*Slc2a5)* expression increased following chronic stress in females but was unaffected in males. (G, H) GLUT8 (*Slc2a8)* decreased in female adult controls, but was unaffected in males. Data shown represent mean fold change $2^{-\Delta CT}$ ± SEM. Different letters indicate significant differences in post-hoc testing with $p<0.05$. C=Control; S=Stress.

Figure 2.5.



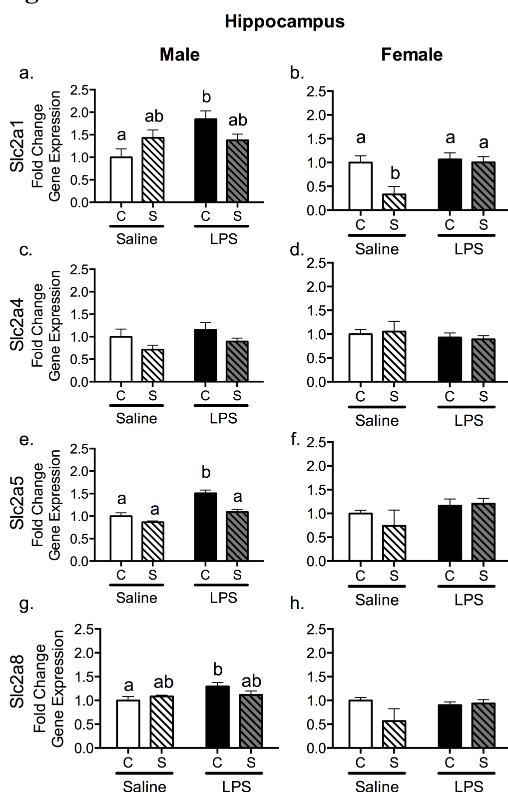
Amygdalar expression of GLUTs is largely impervious to sex, age, and stress. (A,B) GLUT1 (*Slc2a1)* amygdalar expression in male and female rats was unaffected. (C,D) Stressed adult males had increased GLUT3 (*Slc2a3)* expression relative to male adolescent controls, but females were unaffected. (E,F) Age and stress impacted GLUT5 (*Slc2a5)* expression in males but not females. (G,H) GLUT8 (*Slc2a8)* expression in the amygdala was impervious to both age and stress in males and females. Data shown represent mean fold change $2^{-\Delta CT}$ ± SEM. Different letters indicate significant differences in post-hoc testing with $p<0.05$. C=Control; S=Stress.

Figure 2.6.



Effects of Adolescent Chronic Stress on Hippocampal GLUT mRNA Abundance Persist into Adulthood in a Sexually Dimorphic Manner a. Four and a half weeks after the end of chronic adolescent stress, sex and stress significantly interacted to affect *Slc2a1* expression in the hippocampus. Post-hoc testing indicated that control female rats had significantly higher *Slc2a1* expression than control males, while stressed female rats had significantly lower *Slc2a1* expression than controls of either sex. b. Sex significantly increased hippocampal *Slc2a4* expression. Post-hoc testing showed that this effect was due to increased relative expression of *Slc2a4* among stressed and control females vs. stressed males. No long-term effect of stress or sex effect was observed for *Slc2a3, Slc2a5*, or *Slc2a8.* Data are shown as mean fold change $2^{-\Delta CT}$ ± SEM. Letters indicate significant differences in post-hoc testing. C=Control; S=Stress.

Figure 2.7



Adolescent Chronic Stress Blunts the Male Increase in Hippocampal GLUT mRNA Abundance after LPS a. LPS influenced *Slc2a1* expression and interacted with stress to alter expression in males. Post-hoc testing demonstrated that LPS injection only elevated *Slc2a1* expression non-stressed males. b. In females, *Slc2a1* expression was affected by both LPS and prior stress history. Post-hoc testing indicated a decrease in *Slc2a1* expression in saline-treated stressed females. e. Both LPS and prior stress affected *Slc2a5* expression in males, and LPS and prior stress also interacted to influence male *Slc2a*5 hippocampal expression. Post-hoc testing demonstrated that LPS elevated *Slc2a5* expression non-stressed males only. f. LPS altered *Slc2a8* expression in males . However, post-hoc testing once more showed that LPS only elevated *Slc2a8* expression non-stressed males. c, d, f, h. *Slc2a4* was unaffected by LPS in both sexes, and *Slc2a5* and *Slc2a8* were unaffected in females. Data are shown as mean fold change $2^{-\Delta CT}$ ± SEM. Letters indicate significant differences in post-hoc testing. C=Control; S=Stress.

Chapter Three:

Estrous Cycle and Ovariectomy Influence Cerebral Glucose Transporter Expression in a Region- and Isoform-Specific Pattern

Adapted from:

Harrell CS, Burgado J, Kelly SD, Neigh GN. Ovarian steroids influence cerebral glucose transporter expression in a region- and isoform-specific pattern. *Journal of Neuroendocrinology.* 2014;26(4):217-25.

3.0 Abstract

Cerebral glucose uptake is mediated by several members of the family of facilitated glucose transporters (protein nomenclature GLUT; gene nomenclature solute carrier family 2 *Slc2a*). Glucose uptake differs between the sexes and also varies with menstrual status in women and across the rodent estrous cycle. This study demonstrates the extent to which hormonal variation across the four stages of the rat estrous cycle affects mRNA abundance of four members of the GLUT family, including the most well characterized cerebral transporters *Slc2a1* and *Slc2a3,* as well as the insulin-sensitive transporters *Slc2a4* and *Slc2a8* in the hypothalamus, hippocampus, and prefrontal cortex. *Slc2a1* varied significantly across the cycle in the hippocampus and prefrontal cortex, and *Slc2a3 and Slc2a4* also showed significant fluctuation in the hippocampus. Transporter expression significantly increased during proestrus in both the hippocampus and prefrontal cortex. Furthermore, ovarian hormones are critical to normal expression of GLUT mRNA as demonstrated by reduced expression of *Slc2a1, Slc2a3, and Sl2a8* in the hippocampus following ovariectomy. Collectively, the data presented here demonstrate that glucose transporters are highly sensitive to hormonal variation and that this sensitivity is regionally distinct; thereby fluctuations likely have specific phenotypic implications.

3.1. Introduction

As discussed in Chapter Two, glucose is the primary fuel of the brain and alterations in glucose availability directly impact neuronal function. Facilitated glucose transport is mediated by a family of transporters (GLUT) responsible for glucose transport across the endothelial cells of the blood brain barrier, and uptake of glucose into astrocytes and neurons (B. S. McEwen & Reagan, 2004; Simpson et al., 2007). The GLUT family is not completely characterized, but the information currently available indicates that at least seven members of the family are expressed in the brain (B. S. McEwen & Reagan, 2004). GLUT1 is essential for glucose uptake across the blood brain barrier (Pardridge, Boado, & Farrell, 1990) and into astrocytes (Vannucci, Maher, & Simpson, 1997), while GLUT3 is the primary neuronal glucose transporter (Duelli & Kuschinsky, 2001; B. S. McEwen & Reagan, 2004). GLUT4 and GLUT8, both insulin-sensitive glucose transporters (Sankar et al., 2002), have only more recently been recognized for their cerebral expression (B. S. McEwen & Reagan, 2004).

Although extreme changes in GLUTs and glucose uptake have pathological implications for brain function and behavior, such as in De Vivo disease, a rare genetic condition in which GLUT1 is not expressed (De Vivo et al., 2002), some level of variation in glucose metabolism and transport are normal. For instance, baseline regional cerebral glucose metabolism differs between males and females (Gur et al., 1995). Neuroimaging studies have demonstrated that both age and sex affect cerebral glucose metabolism (Kim, Kim, & Kim, 2009). In Chapter Two, we demonstrated that age and sex likewise impact cerebral GLUT expression (Kelly, Harrell, & Neigh, 2013). Glucose uptake appears to be highly plastic with fluctuations evident over the rat estrous cycle – a cycle that repeats every four to five days. Ovarian steroids may mediate alterations in glucose uptake as the highest glucose uptake occurs during pro-estrus (Nehlig, Porrino, Crane, & Sokoloff, 1985), and cerebral glucose metabolism decreases in postmenopausal non-users of estrogen replacement therapy (Rasgon et al., 2005).

Despite the evidence of hormonally induced fluctuations in glucose uptake, the effects of ovarian steroids on expression of glucose transporters have not been fully addressed. In this study, we determined the effects of the estrous cycle hormonal fluctuation on expression of several *Slc2* family genes (solute carrier family 2, the gene family for facilitated glucose transporters). We hypothesized that the increases in estradiol and progesterone, which peak in subsequent order in pro-estrus (Butcher, Collins, & Fugo, 1974), would increase GLUT gene expression, and we focused our assessment on four members of the family likely to respond to ovarian steroids **(Table 3.1)** (Barros, Machado, Warner, & Gustafsson, 2006; N. A. Goldman et al., 2006; X. Li et al., 2013; B. S. McEwen & Reagan, 2004; Shi & Simpkins, 1997). Given the variable influence of the estrous cycle on brain region function, we hypothesized that GLUT gene expression would be differentially impacted by the estrous cycle in the hippocampus, prefrontal cortex, and hypothalamus. These regions were selected based on the role of the hypothalamus in both hormonal (Barrett, Barman, Boitano, & Brooks) and metabolic (Levin, Dunn-Meynell, & Routh, 1999) regulation, the known responsiveness of both hippocampal (Woolley, 1998) and prefrontal cortex (Keenan, Ezzat, Ginsburg, & Moore, 2001) to sex steroids, and the known regulatory circuitry among these regions (Herman & Cullinan, 1997; Sapolsky, Armanini, Packan, Sutton, & Plotsky, 1990). Given that our initial results strongly suggested a role of ovarian steroids in control of the expression of GLUTs in the hippocampus, we ovariectomised a subset of rats in order to conclusively determine the impact of ovarian steroids on hippocampal gene expression of GLUTs.

Collectively, the data presented here demonstrate that glucose transporters are highly sensitive to hormonal variation and that this sensitivity is regionally distinct and thereby fluctuations likely have specific phenotypic implications. Establishment of a framework of understanding regarding the relationship between regional brain metabolism and ovarian hormone influence is critical to assessment of the potential role of GLUTs in ovarian hormone dependent

changes in behavior, consequences of disease states, and the interplay of ovarian senescence, behavior, and aging.

3.2. Materials and Methods

3.2.1 Animal Husbandry For the first experiment evaluating GLUT expression across the estrous cycle, timed pregnant Wistar rats (n=16) were obtained on gestational day 12 from Charles River (Wilmington, MA). Rats were housed on a 14:10 reverse light: dark cycle (lights on: 12 am; lights off: 2pm) in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). Pups were culled on postnatal day (PND) 3 to litters of eight per dam (balanced for sex) and weaned on PND 23, then maintained on standard chow (Lab Diet 5001) until adulthood (PND 60). Only female offspring were used in the current study. No more than two pups from any one litter were assigned to an experimental group in order to control for litter effects; a total of 49 females were used. For the second experiment evaluating GLUT expression after ovariectomy, female Wistar rats (PND56-60) were obtained from Charles River and housed in the same conditions as the rats in the first experiment (n=8 per group). All experiments were approved by the Institutional Animal Care and Use Committee of Emory University and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

3.2.2 Cycle Staging Beginning in adulthood between PND 60 and PND 70, vaginal lavage was performed between 10am and 1pm each day. Cycle was assessed as diestrus 1 (D1), diestrus 2 (D2), pro-estrus (P) or estrus (E) based on the methods described by Goldman et al, 2007 (J. M. Goldman et al., 2007) and Westwood, 2008 (Westwood, 2008). Briefly, D1 was typified by leukocytes and sparse round epithelial cells; D2 by the presence of numerous leukocytes with rare larger round epithelial cells; P by the presence of round, nucleated, granular epithelial cells; and E by the presence of needle-like keratinized epithelial cells among more

rounded cells. Rats were selected over several days, selecting two to three rats daily per cycle stage. In order to insure proper staging, only the rats with regular four-day cycles in which cytology, uterine weight, and plasma estradiol and progesterone concentrations aligned to indicate cycle stage were used in the final analyses for a final n=32. All rats were rapidly decapitated between 10 am and 12 pm, corresponding to between 2-4 hours prior to the end of their light cycle. Rats were collected from the various groups in a counterbalanced manner to guard against circadian effects. Trunk blood was collected for plasma estradiol and progesterone measurements, uteri were collected to correlate uterine weight to cytologic staging, and brains were removed and flash frozen on dry ice before storing at -80°C. The hippocampus, prefrontal cortex, and hypothalamus were dissected from these animals and stored at -80°C until homogenized for gene analysis.

3.2.3 Plasma Hormone Analysis The plasma sex steroids progesterone (sensitivity: 8.57 pg/ml, Enzo Life Sciences) and estradiol (sensitivity: 19 pg/mL, Cayman Chemical, Ann Arbor, MI, USA) were analyzed using enzyme-linked immunoassay. Samples were run in duplicate for all endocrine assays.

3.2.4 Ovariectomy After acclimatization to the animal facility, ovariectomy (n=8) or a sham operation (n=8) was performed on the adult female Wistar rats. Rats were dosed 100ul per 100g rat with a ketamine/xylazine/acepromazine cocktail (25:5:1; original concentrations: ketamine 50mg/ml; xylazine 20mg/ml; acepromazine: 10mg/ml) to induce anesthesia and surgery was performed after the rat reached an anesthetic plane. Rats were maintained under anesthesia with inhaled isoflurane. Each flank was shaved to the hip and cleaned with betadine and alcohol, and an abdominal incision was made to locate the ovaries. For the ovariectomies, ovaries were gently pulled out, ligated with 3-0 silk suture, and cut. For all animals, the abdominal cavity was closed with 5-0 absorbable suture, and surgical staples were used to remove the skin. Betadine was applied to the staples, and the rats were placed on a heating pad and monitored. Both sham-operated and ovariectomized rats received fruit loops and oral meloxicam for one day, and

remained under daily observation until staple removal five days post-operation. Sham surgeries were conducted identical to ovariectomy with the exception of the ligation and removal of the ovaries. Rats were rapidly decapitated seven days after ovariectomy or sham surgery, and plasma and brain were collected as described above.

3.2.5 Quantitative RT- PCR Hippocampal RNA was extracted via RNeasy Mini kit (Qiagen, Valencia, CA, USA). The RNA integrity was then assessed with a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA). Using the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA), RNA was reverse transcribed and subsequently cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized to 1 ng/ul. This allows for standardizing at two separate times: once prior to reverse transcription, and again prior to RT-PCR. Rat TaqMan Gene Expression Assays were purchased from Applied Biosystems (Carlsbad, CA, USA) with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively: *Slc2a1* (Rn01417099_m1), *Slc2a3* (Rn00567331_m1), *Slc2a4* (Rn00562597_m1), and *Slc2a8* (Rn00585203_m1). The following two-step RT-PCR cycling conditions were used on the 7900HT Sequence Detection System (Applied Biosystems): 50° C (2 min), 95° C (10 min), 40 cycles of 95° C (15 s) and 60° C (1 min). The housekeeping genes, *Ubc* (Rn01789812_g1), *Hprt1* (Rn01527840_m1), and *Tfrc* (Rn01474701_m1), were run as potential endogenous controls. However, when analyzed either separately or using the geometric means of the cycle threshold values (calculated as described in (Vandesompele et al., 2002), housekeeping gene expression varied by cycle stage, which could confound analysis of the genes of interest. Relative gene expression of individual samples run in triplicate (with coefficient of variation cut-off set to 4%) was thus determined by the comparative C_T quantification method only relative to either diestrus 1 or sham ($2^{-\Delta CT}$). Additional precautions of double standardization were made to ensure consistent loading in the RT-PCR: once of the RNA prior to reverse transcription, and again of the cDNA prior to RT-PCR. All TaqMan gene expression assays are guaranteed to have 90 - 100% amplification efficiency as determined by the

genome-aided probe and primer design pipeline and reported in the "Amplification Efficiency of TaqMan Gene Expression Assays" Application Note 127AP05-03 from Applied Biosystems.

3.2.6 Statistical Analysis Statistical comparisons of differences in gene expression and hormone concentrations were analyzed using GraphPad Prism 6.0 Statistical Software. Hormone concentrations and comparative C_T quantification was analyzed by One-Factor ANOVA followed by Holm-Sidak post-hoc testing in experiment one (using the factor of cycle stage), and by unpaired two-sided Student's t-test in experiment two (sham vs. ovariectomized). Outliers were assessed by the Grubb's Outlier Test and removed. Data were determined to be statistically significant when $p < 0.05$ and are presented in the figures as mean ± standard error of the mean (SEM).

3.3. Results

3.3.1 Estrous cycle stage was established from vaginal lavage and confirmed with uterine weights and hormone concentrations. Uterine weight was normalized to each rat by dividing uterine weight (in mg) by the rat weight (in g). Normalized uterine weight varied significantly across the estrous cycle ($F_{3, 28}=27.35$; $p<0.0001$), with the greatest uterine weight in pro-estrus (vs. D1: $t_{28}=8.308$, $p<0.0001$; vs. D2: $t_{28}=6.796$, $p<0.0001$; vs. E: $t_{28}=5.176$, $p<0.0001$; **Figure 3.1a**), as expected based on a review of the literature (Goodman, 1978; Nequin, Alvarez, & Schwartz, 1979). The ratio of progesterone (P4; in ng/ml) to estradiol (E2; in pg/ml) was calculated for all samples for which the sex steroid levels fell within the range of the ELISA assay and with the appropriate coefficient of variation for both hormones. This ratio reflects the hormonal state at the end of light cycle for all animals, as decapitation and collection occurred for all animals between two and four hours prior to the beginning of the dark cycle. The ratio varied significantly across the estrous cycle ($F_{3, 12}=12.19$; $p=0.0006$; **Figure 3.1b**), with the highest P4:E2 ratio in pro-estrus (vs. D1: $t_{12}=4.564$, $p=0.0026$; vs. D2: $t_{12}=5.357$, $p=0.0010$; vs. E:

t_{12}=5.176, p=0.0012), as expected due to the fact that samples were collected late in the light cycle or just before the dark cycle during pro-estrus, and thus would be after the estradiol surge and during the progesterone surge (Butcher et al., 1974; Kalra & Kalra, 1974; Nequin et al., 1979).

3.3.2 The hippocampus and hypothalamus demonstrate a greater relative abundance of GLUT mRNA than the prefrontal cortex. Relative gene expression in D1 of each GLUT across the three regions studied (hippocampus, prefrontal cortex, and hypothalamus) revealed significant regional variation for all GLUT genes analyzed: *Slc2a1*, $F_{2, 15}$=10.13 and p=0.0016; *Slc2a3*, $F_{2, 15}$=5.867 and p=0.0131; *Slc2a4*, $F_{2, 15}$=110.6 and p<0.0001; and *Slc2a8*, $F_{2, 15}$=380.4 and p<0.0001 (**Figure 3.2)**. Post-hoc testing showed that GLUT gene expression was lowest in the prefrontal cortex for all glucose transporters analyzed (*Slc2a1*: PFC vs. HP: t_{15}=3.179, p=0.0124; PFC vs. HYT: t_{15}=4.350, p=0.0017; *Slc2a3:* PFC vs. HP: t_{15}=3.149, p=0.0197; *Slc2a4:* PFC vs. HP: t_{14}=2.620, p=0.0202; vs. HYT: t_{14}=14.12, p<0.0001; *Slc2a8*: PFC vs. HYT: t_{15}=24.19, p<0.0001). Analysis of each region separately across all four glucose transporters in D1 demonstrated that further significant isoform-specific variation existed throughout the brain: hippocampus, $F_{3, 20}$=6.154 and p=0.0039; prefrontal cortex, $F_{3, 20}$=30.06 and p<0.0001; and hypothalamus, $F_{3, 19}$=96.52 and p<0.0001 **(Figure 3.3**). *Slc2a1* expression was greatest and *Slc2a4* expression was lowest in all three regions (HP: *Slc2a1 vs. Slc2a4:* t_{20}=3.786, p=0.0069; *Slc2a1* vs. *Slc2a8* t_{20}=3.620, p=0.0085; PFC: *Slc2a1 vs. Slc2a3:* t_{20}=3.643, p=0.0048; *Slc2a1 vs. Slc2a4:* t_{20}=9.045, p<0.0001; *Slc2a1 vs. Slc2a8:* t_{20}=6.439, p<0.001; *Slc2a4 vs. Slc2a3:* t_{20}=5.402, p<0.0001; *Slc2a4 vs. Slc2a8:* t_{20}=2.602, p=0.0222; HYT: *Slc2a1 vs. Slc2a3:* t_{19}=12.72, p<0.0001; *Slc2a1 vs. Slc2a4:* t_{19}=12.76, p<0.0001). While the hippocampus and prefrontal cortex showed the same general pattern, with gene expression decreasing from *Slc2a1* to *Slc2a3* to *Slc2a8* to *Slc2a4*, the hypothalamus had a different pattern, with gene expression highest in *Slc2a1* and *Slc2a8* and lower in *Slc2a3* and *Slc2a4*.

3.3.3 The hippocampus and prefrontal cortex demonstrate greater cycle-dependent variation in gene expression for GLUTs than the hypothalamus. When analyzed by a one-way ANOVA within each region and normalizing to D1, relative gene expression of *Slc2a1* varied by cycle stage in both the hippocampus ($F_{3, 26}$=4.765; p=0.0089; **Figure 3.4**) and the prefrontal cortex ($F_{3, 28}$=4.050; p=0.0165), but not in the hypothalamus ($F_{3, 26}$=0.6155; p=0.6114). Relative gene expression of *Slc2a3* ($F_{3,25}$=4.110; p=0.0168) and *Slc2a4* ($F_{3,25}$=3.965; p=0.0193) also varied significantly across the estrous cycle within the hippocampus (**Figure 3.5)** but expression of these transporters did not fluctuate in the prefrontal cortex (*Slc2a3:* $F_{3,26}$=2.107; p=0.1219; *Slc2a4:* $F_{3,26}$=2.378; p=0.0910) or the hypothalamus (*Slc2a3:* $F_{3,26}$=1.269; p=0.3056; *Slc2a4:* $F_{3,26}$=0.8541; p=0.4788). *Slc2a8* expression did not vary by cycle stage in any region analyzed (HP: $F_{3, 26}$=2.575 p=0.0765; PFC: $F_{3, 26}$=2.374; p=0.0915; HYT: $F_{3, 26}$=0.03024; p=0.9928).

3.3.4 Expression of GLUT1, 3, and 8 in the hippocampus is directly influenced by ovarian hormones. In order to determine whether ovarian hormones were necessary for baseline GLUT expression in the hippocampus, the brain region that demonstrated the greatest variation in GLUT expression over the estrous cycle, female rats were ovariectomized prior to assessment of GLUT gene expression in the hippocampus. Removal of ovarian hormones was sufficient to cause a reduction in hippocampal gene expression for *Slc2a1* (t_{13}=5.581; p<0.0001), *Slc2a3* (t_{12}=4.231; p=0.0012), and *Slc2a8* (t_{13}=7.711; p=<0.0001), but not *Slc2a4* (p=0.9692); **Figure 3.6**).

3.4 Discussion:

In diestrus, expression of GLUT gene isoforms varied between brain regions in female rats. *Slc2a1*, which encodes for GLUT1 and is expressed in micro vessels (Pardridge et al., 1990) and glial cells (Vannucci et al., 1997), was enriched in hippocampal and hypothalamic tissue (**Figure 3.2**), and more highly expressed than any other glucose transporter in all regions (**Figure 3.3**). This regional distribution agrees with several prior reports of widespread GLUT1

expression in the rat (Maurer, Canis, Kuschinsky, & Duelli, 2004; Vannucci et al., 1997). Given the high vascularization of both the hippocampus and the hypothalamus (Daniel, 1966; Marinkovic, Milisavljevic, & Puskas, 1992) it is not surprising that these regions would be particularly enriched in a blood-brain-barrier glucose transporter. Although previous reports suggest greater GLUT activity in the prefrontal cortex than is implied by the current findings of low GLUT gene expression, existing literature on GLUT expression in the cortex has either examined the cortex broadly (Vannucci, 1994) or specified different subregions (Duelli et al., 2000) than those observed in the current study. One potential explanation for this lower GLUT expression in the prefrontal cortex than the hypothalamus or hippocampus is that the region is relatively less vascularised. To this end, exercise is able to induce a stimulatory effect on hippocampal but not prefrontal angiogenesis (Ekstrand, Hellsten, & Tingstrom, 2008), consistent with potential regional vascular differences.

The high hypothalamic expression during diestrus 1 of both *Slc2a4* and *Slc2a8*, in addition to *Slc2a1*, is notable. The hypothalamus is a region of both critical hormonal and metabolic control (Levin et al., 1999), and estradiol in particular is known to act in the ventromedial hypothalamus to decrease food intake (Butera, 2010). The current findings agree with prior reports of GLUT4 expression in the hypothalamus (Kobayashi, Nikami, Morimatsu, & Saito, 1996; Ngarmukos, Baur, & Kumagai, 2001). GLUT8, with high hypothalamic expression in the current study, has also been reported in the hypothalamus (Sankar et al., 2002) though this glucose transporter is less well characterized than the other three studied in this series of experiments due to its more recent discovery (B. S. McEwen & Reagan, 2004). The relatively high expression of GLUT1 (Chari et al., 2011) may be related to the role of the hypothalamus in glucose-sensing and glucose production and may correspond with the hypothalamus' role in metabolic regulation.

In addition to determining cerebral regional and isoform-specific GLUT expression during a single stage of the estrous cycle, the current experiment established the existence of

important fluctuations in transporter expression across the estrous cycle. *Slc2a1* expression varies over the estrous cycle in both the hippocampus and the prefrontal cortex, without similar changes in the hypothalamus. The absence of an effect on hypothalamic GLUT expression is in contrast to previous findings. Specifically, Nehlig, Porrino et al (1985) reported changes in hypothalamic glucose uptake, with greatest increases in pro-estrus (Nehlig et al., 1985). However, they report this increase in only five of the fifteen hypothalamic subregions analyzed (Nehlig et al., 1985). Thus, the current analysis assessing global hypothalamic gene expression may not have assessed the smaller fluctuations apparent in a more subregion specific analysis.

Unlike its lack of effect in the hypothalamus, the stage of the estrous cycle influenced both the hippocampus and prefrontal cortex, such that *Slc2a1* expression peaked in pro-estrus and was significantly lower in estrus. These data resemble previously reported changes in cerebral glucose uptake across the estrous cycle (Nehlig et al., 1985). Nehlig, Porrino et al (1985) reported fluctuations in glucose uptake for the entorhinal cortex and CA3 region of the hippocampus, with peaks in pro-estrus as well as metestrus (synonymous with diestrus 1; (J. M. Goldman et al., 2007), and decreases in diestrus 2 and estrus (Nehlig et al., 1985), similar to the pattern seen in these studies for *Slc2a1* and *Slc2a3* in the hippocampus. The pattern dissimilarity between the hypothalamus and hippocampus is intriguing given the rich blood supply and hormonal access of both regions. However, these regions have different relative densities of progesterone (Guerra-Araiza, Cerbon, Morimoto, & Camacho-Arroyo, 2000) and estrogen receptors (Simerly, Chang, Muramatsu, & Swanson, 1990), which have also been shown to vary in expression in response to ovarian hormones (Guerra-Araiza et al., 2000; Simerly, Carr, Zee, & Lorang, 1996). This variable receptor density could impact the hormonal effect on fluctuations in GLUT expression. Differential expression of receptor and receptor isoforms and/or different regional sensitivity to hormones (Guerra-Araiza et al., 2000; Simerly et al., 1990) may play a role in the lack of variation observed across the estrous cycle.

In addition to changes in *Slc2a1* expression, the hippocampus also showed altered *Slc2a3* and *Slc2a4* expression over the estrous cycle. *Slc2a3* expression followed a similar pattern to *Slc2a1*, as did *Slc2a8* (although *Slc2a8* expression changes were not significant). These findings are consistent with prior reports of increases in GLUT expression in response to ovarian hormone administration. Estradiol replacement has previously been shown to affect glucose uptake into the rodent brain (Bishop & Simpkins, 1995) and also to increase GLUT1 expression in blood-brain-barrier endothelial cells (Shi & Simpkins, 1997) as well as GLUT3 and GLUT4 expression in primate cortex (Cheng, Cohen, Wang, & Bondy, 2001). Additionally, progesterone may play a key role influencing GLUT1 expression in the hippocampus. Progesterone administration has previously been shown to increase GLUT1 expression in rat endothelial cells and GLUT3 expression in rat hippocampus (X. Li et al., 2013), and increases in glucose uptake have been shown in this region during metestrus and pro-estrus (Nehlig et al., 1985). As seen in Figure 1, and consistent with previous reports (Nequin et al., 1979; Sodersten & Eneroth, 1981), there is slight surge in the progesterone to estradiol ratio in D1 in addition to the large surge in the P4:E2 ratio during pro-estrus, due to the fact that all samples were collected toward the end of the light cycle. It is additionally remarkable the elevations in hippocampal GLUT1 occurred in both diestrus and pro-estrus, offering supporting evidence for the regulatory role of progesterone in GLUT1 expression.

In contrast, hippocampal *Slc2a4* showed a distinct pattern from *Slc2a1* and *Slc2a3* across the estrous cycle, with significant increases in estrus relative to diestrus 1. GLUT4 is an insulin sensitive transporter, and estradiol is known to play a significant role in modulating peripheral insulin sensitivity (Barros et al., 2006) as well as promoting GLUT4 expression in muscle and adipocytes (Im, Kwon, Kim, Kim, & Ahn, 2007). Progesterone, on the other hand, can promote insulin resistance in various tissues (Livingstone & Collison, 2002). Our pro-estrus samples were taken during the surge of progesterone, and the failure to see to an increase in *Slc2a4* during pro-estrus may be due to the relative ratio and divergent effects of the ovarian hormones in the rat.

The fact that the hippocampus, relative to the prefrontal cortex and hypothalamus, experienced the greatest changes in glucose transporter expression correlates with known changes in hippocampal structure as well as behavioral changes that occur across the estrous cycle and in response to ovarian hormone administration. Both exogenous administration of estradiol and progesterone and fluctuation of these ratios across the estrous cycle affect hippocampal spine density, with increases in estradiol associated with preservation of spine density and progesterone associated with a decrease in spine density (Woolley & McEwen, 1993). Pro-estrus is also associated with altered behavior, including reduced anxiety-like behavior and improved performance in hippocampal-dependent tasks (Walf, Koonce, Manley, & Frye, 2009; Woolley, Gould, Frankfurt, & McEwen, 1990). Changes in hippocampal GLUT3, GLUT4, and GLUT8 expression or function have also been associated with either dexamethasone administration (Piroli et al., 2007) or stress exposure (Piroli et al., 2004; Reagan et al., 2000). Increased cerebral glucose uptake, mediated either by natural fluctuations in ovarian hormone (Nehlig et al., 1985) or exogenous administration of ovarian hormones (Bishop & Simpkins, 1995) may be mediated by changes in hormonally-dependent GLUT expression (Figures 3.4, 3.5, and 3.6; . These changes in glucose availability may underlie behavioral and synaptic changes mediated at the level of the hippocampus.

Given the particularly complex degree of changes in GLUT gene expression in the hippocampus over the estrous cycle, we directly examined the influence of sex steroids on GLUT gene expression in the hippocampus by removing ovarian hormones. As expected based on the estrous cycle data, ovariectomy reduced expression of *Slc2a1*, *Slc2a3*, and *Slc2a8*. The lack of change in *Slc2a4* may be due to the fact that it is already expressed at low levels, and therefore may approach a bottoming-out effect at which the technique used is unable to appreciate further reductions. Alternatively, *Slc2a4* may be differentially responsive to ovarian hormones, particularly influenced by estradiol (Ruegg et al., 2011), and as the data indicated by its differential pattern of expression across the estrous cycle. These changes in GLUT expression

across the estrous cycle and after removal of ovarian hormones are particularly relevant given the potential impact of surgical menopause-exacerbated and menopause-related cognitive decline (Farrag, Khedr, Abdel-Aleem, & Rageh, 2002; Rocca et al., 2007). This interest has sparked research into the potential mechanisms whereby ovarian hormonal modulates neural function (B. McEwen, 2002). The data presented here point towards the important modulatory role that ovarian hormones play in cerebral GLUT expression, and together with the greater literature and clinical findings indicate a need for future studies to better understand the precise nature of this relationship and its therapeutic implications.

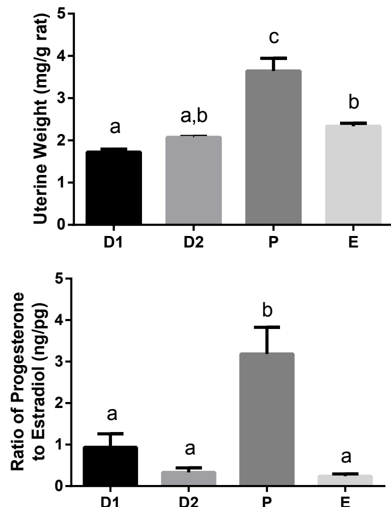
In sum, the current set of experiments demonstrated regional and isoform-specificity of cerebral GLUT gene expression in the female rat during a single stage of the estrous cycle. When expression across the estrous cycle was assessed, the hippocampus was particularly susceptible to the effects of fluctuating ovarian steroids, which is consistent with cyclic changes in hippocampal-dependent behavior and structure. In addition, removal of ovarian hormones via ovariectomy reduced expression of glucose transporters in the hippocampus. These studies demonstrate that ovarian steroids are essential modulators of glucose transporter expression in limbic brain regions, and indicate a need for further research to explore the impact of such modulation on neural function.

Table 3.1

Glucose Transporter	Gene Name	Cerebral Cell Type	Functional Notes
GLUT1-45kDa	*Slc2a1*	Astrocytes, neurons (?)	Astrocyte glucose transport
GLUT1-55kDa		Endothelial cells	Blood-brain barrier glucose transport
GLUT3	*Slc2a3*	Neurons (somatodendritic)	Primary neuronal glucose transporter
GLUT4	*Slc2a4*	Neurons (somatodendritic)	Insulin-stimulated glucose transport via plasma membrane
GLUT8	*Slc2a8*	Neurons (somatodendritic)	Insulin-stimulated translocation to endoplasmic reticulum

This table summarizes the main functions of the cerebral glucose transporter isoforms analyzed with respect to ovarian hormones.

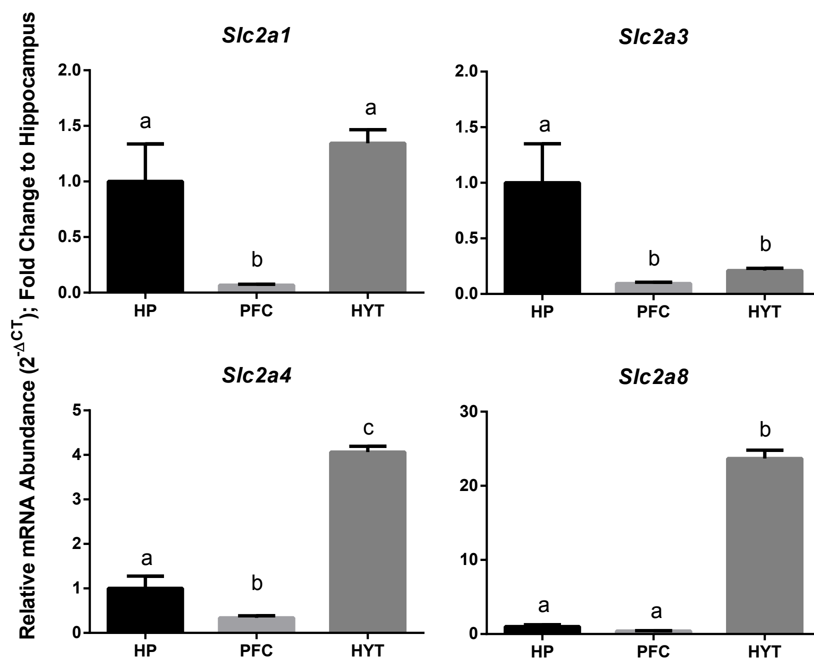
Figure 3.1.



Uterine weight and P:E ratio fluctuate across estrous cycle stages.

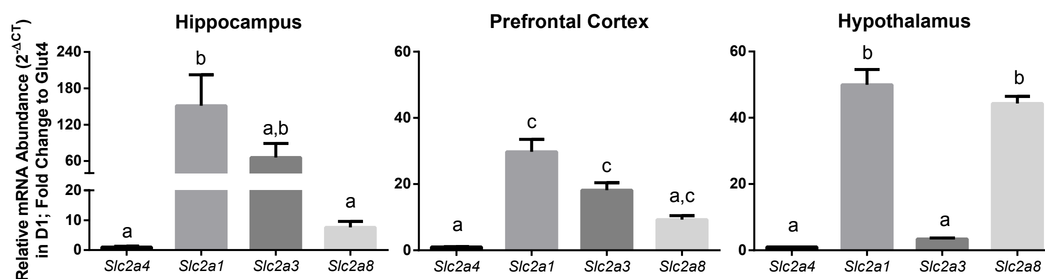
(a) Uterine weight was normalized to each rat by taking the uterine weight (in mg) and dividing by the rat weight (in g). Normalized uterine weight varied significantly across the estrous cycle, with the greatest uterine weight in pro-estrus. **(b)** The ratio of plasma progesterone (P4; in ng/ml) to plasma estradiol (E2; in pg/ml) varied significantly across the estrous cycle, with the highest P4:E2 ratio in pro-estrus. Data are presented as mean ± SEM. Letters indicate significant post-hoc differences, with different letters indicating significant differences ($p<0.05$).

Figure 3.2.



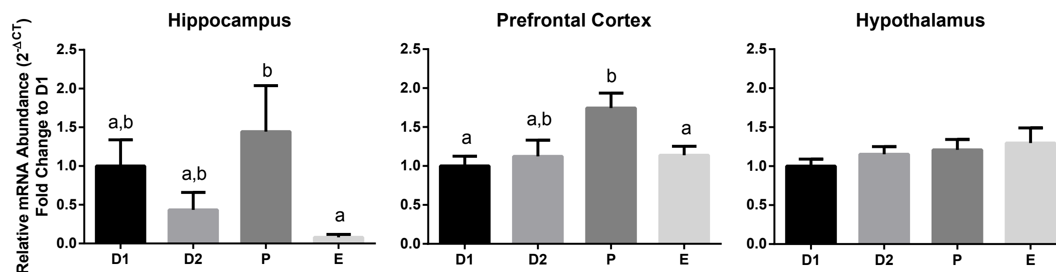
The hippocampus and hypothalamus demonstrate a greater relative abundance of GLUT mRNA than the prefrontal cortex. Fold change in gene expression in diestrus 1 (D1) of each glucose transporter was calculated by normalizing to hippocampal expression and analyzed by a One-Way ANOVA. Analysis revealed significant regional variation for all genes analyzed: **(a)** *Slc2a1*, **(b)** *Slc2a3*, **(c)** *Slc2a4*, and **(d)** *Slc2a8*. Holm-Sidak post-hoc testing showed that glucose transporter gene expression was lowest in the prefrontal cortex for all glucose transporters analyzed. Data are presented as mean ± SEM. Letters indicate significant post-hoc differences, with different letters indicating significant differences ($p<0.05$).

Figure 3.3.



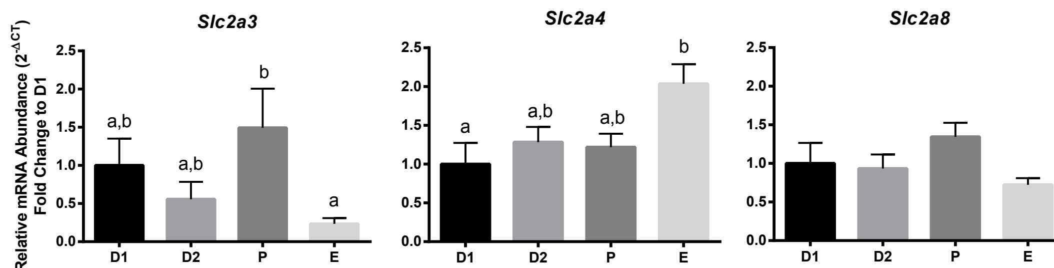
GLUT1 mRNA abundance is greatest and GLUT4 mRNA abundance is lowest in all regions analyzed. Fold change in regional gene expression of glucose transporters in diestrus 1 (D1) was calculated by normalizing to *Slc2a4* and analyzed by One-Way ANOVA. Analysis demonstrated that significant isoform-specific variation existed throughout the brain. Shown are: **(a)** hippocampus (HP), **(b)** prefrontal cortex (PFC), and **(c)** hypothalamus (HYT). *Slc2a1* expression was greatest and *Slc2a4* expression was lowest in all three regions. Data are presented as mean ± SEM. Letters indicate significant Holm-Sidak post-hoc differences, with different letters indicating significant differences ($p<0.05$).

Figure 3.4.



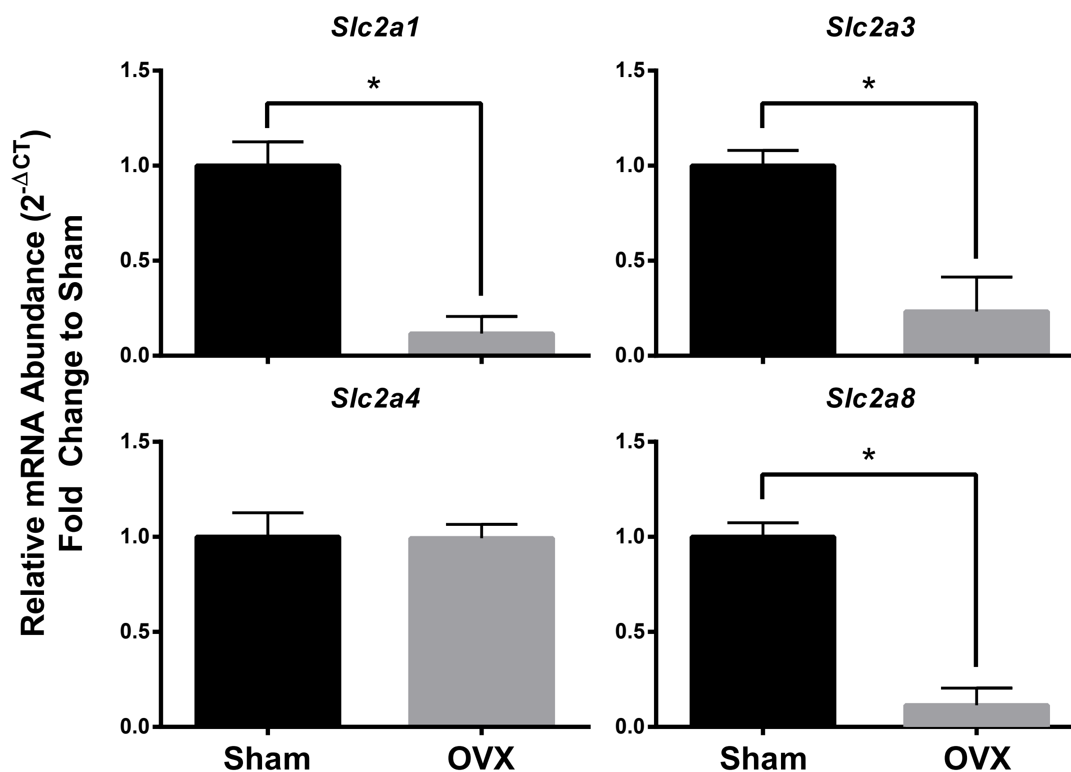
The hippocampus and prefrontal cortex demonstrate greater cycle-dependent variation in gene expression for GLUT1 than the hypothalamus. Fold change in regional expression of *Slc2a1* was calculated by normalizing to diestrus 1 (D1) and analyzed by One-Way ANOVA. Analysis revealed significant variation by cycle stage in both **(a)** the hippocampus and **(b)** the prefrontal cortex but not in **(c)** the hypothalamus. Data are presented as mean ± SEM. Letters indicate significant Holm-Sidak post-hoc differences, with different letters indicating significant differences ($p<0.05$).

Figure 3.5.



Hippocampal mRNA abundance of GLUT3 and GLUT4 varies by cycle stage and peaks in pro-estrus and estrus respectively. Fold change in hippocampal glucose transporter gene expression was calculated compared to diestrus 1 (D1) and analyzed by One-Way ANOVA. Relative gene expression of **(a)** *Slc2a3* and **(b)** *Slc2a4* varied significantly across the estrous cycle, but **(c)** *Slc2a8* expression did not vary by cycle stage. Data are presented as mean ± SEM. Letters indicate significant Holm-Sidak post-hoc differences, with different letters indicating significant differences ($p<0.05$).

Figure 3.6.



Ovarian hormones directly influence expression of GLUT1, 3, and 8 in the hippocampus. Fold change in hippocampal glucose transporter gene expression of ovariectomized female rats (OVX) was calculated compared to sham one week after surgeries and analyzed by an unpaired two-tailed Student's t-test. Relative gene expression was decreased after ovariectomy for **(a)** *Slc2a1*, **(b)** *Slc2a3*, and **(d)** *Slc2a8*, but not **(c)** *Slc2a4*. Data are presented as mean ± SEM. Asterisks indicate significant differences ($p<0.05$).

INTERLUDE

Chapter Four

Contributors to Cerebral Energy Metabolism

4. Abstract

In Chapters Two and Three, we observed that extracerebral factors, including psychosocial stress, inflammatory stimuli, or hormonal fluctuations, were sufficient to shift cerebral glucose transporter isoform expression in a region specific manner. While the direct effect on cerebral glucose metabolism remains to be determined, we propose that psychosocial stress and inflammatory stimuli may influence cerebral energy homeostasis, and this influence can be modulated by sex hormones. However, it is similarly possible that a direct perturbation of energy homeostasis (such as through dietary modification) may alter the stress response, as discussed in Chapter One. In this interlude, we will review the sources of cerebral metabolic fuel and the way in which a change in metabolic fuel source could alter energy homeostasis.

4.1 Review:

As discussed in Chapters Two and Three, glucose has long been considered the primary fuel for the brain (Sokoloff, 1989), and increased glucose utilization is associated with functional activation of neural issue (Sokoloff, 1999). Sodium ion efflux and potassium ion influx associated with neuronal action potential spiking stimulates Na^{+}/K^{+} ATPase activity, which requires energy metabolism to return to homeostatic resting levels. Glia, and astrocytes in particular, are also major utilizers of glucose, and potentially use glucose at a greater rate (Magistretti & Pellerin, 1999). Demonstrating the critical need for glucose, glucose deprivation for three to four hours will destroy approximately half of all neurons in a glia-confluent cortical culture (Swanson & Choi, 1993).

However, glucose is not the only source of metabolic fuel for the brain. Growing evidence supports the concept of an astrocyte-neuron lactate shuttle (Pellerin et al., 1998), in which glucose is readily taken up into astrocytes through GLUT1 in their end-feet, which cover virtually all intraparenchymal capillaries. Glucose can then be stored in astrocytes as glycogen until needed (Swanson & Choi, 1993). During neuronal activity, synaptic glutamate is transported into astrocytes, and this stimulates anaerobic glycolysis from glucose or glycogen to produce lactate. Lactate is then shuttled into the synaptic cleft, where it is taken up into neurons by monocarboxylate transporters (MCTs) to support activity (Pellerin et al., 1998; Tabernero, Medina, & Giaume, 2006). Though the extent to which this alternative energy source is used versus direct neuronal glucose uptake is still under debate (Chih & Roberts Jr, 2003), it demonstrates the potential for alternative simple sugars to serve as cerebral energy sources.

Ketone bodies are an additional important energy source, particularly during early development (Nehlig, 1996; Patel, 1974). The term "ketone bodies" refers to three molecules derived under different metabolic conditions that transport energy from the liver to other tissues, including the brain. As one might expect, each contains a ketone, or double-bonded oxygen between two carbon-containing substituents (Botham & Mayes, 2012). The first ketone body, acetoacetate, forms from fatty acid metabolism under low carbohydrate conditions (such as starvation, exercise, diabetes, or excessive alcohol consumption) and the second, 3-β-hydroxybutryate, is formed by reduction of acetoacetate in mitochondria. Acetone is formed by spontaneous decarboxylation of acetoacetate and is the ketone body that results in the sweet odor on the breadth of individuals with ketoacidosis (Laffel, 1999). Unlike most tissues, the brain is unable to use fatty acids, and it uses ketone bodies as an alternative energy source (providing up to 2/3 of the brain's energy needs) during periods of prolonged fasting. While this spares glucose utilization and reduces proteolysis, it also generates oxygen radical and induces lipid peroxidation, which may exacerbate vascular disease and diabetes (Laffel, 1999).

Fructose is a simple ketonic monosaccharide with the same molecular formula ($C_6H_{12}O_6$) as its cousin, glucose, an aldose monosaccharide (Bender & Mayes, 2012). They differ in structure due to the ketone (double-bonded oxygen between two carbon atom) in fructose versus the aldehyde (double-bonded oxygen between one carbon and one hydrogen atom) in glucose (see Figure 4.1). While this ketone does not imply that fructose can act as a ketone body, it does necessitate different absorptive and metabolic pathways for fructose versus glucose (Havel, 2005; Sun & Empie, 2012). Fructose is absorbed into the duodenum both passively and by GLUT5, while glucose is absorbed by the sodium/glucose cotransporter-1. Both are then reabsorbed into the bloodstream by GLUT2, though fructose absorption and reabsorption is less efficient than that of glucose (Havel, 2005; Sun & Empie, 2012). Subsequently, glucose is released and widely used peripherally as well as delivered to the liver. Over 60% of fructose, on the other hand, is delivered to the liver, where it is transported into cells by GLUT2 and converted to glucose, lactate, glycerol, glycogen, and lipids, then stored or released; the remaining 30-40% is available immediately for extrahepatic metabolism and reuptake into tissue by GLUT5 (Sun & Empie, 2012).

Interest in fructose metabolism has grown over the past several decades as consumption of fructose has significantly increased. In 1977-1978, fructose consumption ranged from 3 g/day among women over 51 years (the lowest consumers) to 28 g/day among men 19-22 years old (the greatest consumers; (Marriott, Cole, & Lee, 2009)). In 1999-2004, fructose consumption ranged from 32 g/day among women over 51 years to 75 g/day among men 19-22 years old (Marriott et al., 2009). However, as a percent of total energy consumption, this represents only a small increase due to the overall increase in consumption between these two time periods (Marriott et al., 2009). Nonetheless, this increase in fructose consumption in particular has been linked to disruptions in energy homeostasis including obesity (Bray, Nielsen, & Popkin, 2004; Tappy & Le, 2010), diabetes (R. J. Johnson et al., 2009; Le et al., 2009), and metabolic syndrome (Elliott, Keim, Stern, Teff, & Havel, 2002; Stanhope et al., 2009). Fructose does not induce the same

satiety signal as glucose since blood glucose is lower after ingestion of a bolus of fructose, and fructose similarly fails to suppress ghrelin or stimulate leptin to promote satiety (Teff et al., 2004). Fructose also promotes oxidative stress, as it has greater reductive capacity than glucose and can stimulate production of reactive oxygen species and subsequent apoptosis through glycation reactions (Kaneto et al., 1996). Short-term fructose consumption increases de novo lipogenesis, while chronic consumption is associated with dyslipidemia and ectopic lipid deposition (Tappy & Le, 2010). Chronic consumption is similarly associated with increased blood pressure, and impaired insulin sensitivity, likely linked to the elevation in circulating fatty acids and triglycerides (Havel, 2005). Excessive plasma lactate (which interferes with urinary urate excretion) and hyperuricemia related to fructose consumption may also contribute to metabolic abnormalities (Tappy & Le, 2010). Collectively, while the direct cause between fructose and impaired energy homeostasis remains unclear, excessive consumption is linked to metabolic abnormalities on multiple levels.

Though the impact of fructose consumption on peripheral metabolism has been well examined, its impact on cerebral energy homeostasis is less well understood. When injected into the bloodstream of rodents at low concentrations (0.42 mM), fructose uptake into the brain is less than 2% while glucose uptake at this same concentration is approximately 33% (Oldendorf, 1971). However, 60 minutes after an injection of a high concentration of fructose (30 mM), brain fructose concentrations will equal typical brain glucose concentrations (approximately 2 mmol/kg) and are more than half brain glucose concentrations after injection of a 30 mM bolus of glucose (Thurston, Levy, Warren, & Jones, 1972). In addition, by products of fructose metabolism, such as lactate, may also affect cerebral energy homeostasis. Lactate is transported across the blood-brain-barrier at approximately half the rate of glucose when injected at low concentrations, and this transport rate is increased with low pH, such as may be caused by lactate-induced hypercapnia (G. M. Knudsen, Paulson, & Hertz, 1991).

The relationship between fructose and lactate may be especially important for cerebral metabolism even once fructose has crossed the blood brain barrier. Using a rat hippocampal slice preparation, Izumi and Zoromski (Izumi & Zorumski, 2009) demonstrated that 10mM fructose could preserve ATP levels and partially restore synaptic potentials that had been depressed in a glucose-deprived state. These effects were blocked by administration of cytochalasin B, a GLUT inhibitor, as well as by administration of α-cyano-4-hydroxycinnamate, a MCT inhibitor. This data indicates that fructose may supply cerebral metabolism through a mechanism similar to the astrocyte neuron lactate shuttle (Izumi & Zorumski, 2009). Though the mechanisms are more difficult to assess in humans, it is clear that fructose and glucose have different effects on cerebral metabolism. In a study of fructose versus glucose-induced changes in magnetic resonance imaging used to assess cerebral blood flow (CBF) as a proxy for metabolism, a fructose bolus caused a greater reduction in hypothalamic CBF, and also reduced CBF in the thalamus, hippocampus, posterior cingulate cortex, and visual cortex (Page et al., 2013). Further, glucose ingestion increased functional connectivity among the hypothalamus, thalamus, and striatum, whereas fructose ingestion only increased connectivity between the hypothalamus and thalamus (Page et al., 2013). Taken together, these studies indicate the potential for fructose, either directly or indirectly, to alter cerebral as well as peripheral energy homeostasis.

In the subsequent several chapters, we will turn our attention to the potential effects that a dietary manipulation in the form of a high-fructose diet can have on energy homeostasis and the stress response. As discussed in Chapter One, perturbations to energy homeostasis can alter the stress response through a number of possible pathways, including direct dietary effects, influences on central and peripheral glucocorticoid signaling, changes in inflammatory responses, and effects on additional neuroendocrine and neuropeptide factors. Though these studies do not aim to determine whether the results are mediated directly by the fructose molecule on neural tissue or indirectly by the effects of fructose on peripheral metabolism, they will shed light on the

behavioral, hormonal, and molecular influences that a high-fructose diet evokes on metabolism and the stress response.

PART TWO:

DIETARY DISRUPTIONS TO ENERGY AVAILABILITY ALTER THE STRESS RESPONSE

Chapter Five

High-fructose diet during adolescent development increases depressive-like behavior and remodels the hypothalamic transcriptome in male rats

Adapted and expanded from:

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5.0 Abstract

Fructose consumption, which promotes insulin resistance, hypertension, and dyslipidemia, has increased by over 25% since the 1970s. In addition to metabolic dysregulation, fructose ingestion stimulates the hypothalamic-pituitary-adrenal (HPA) axis leading to elevations in glucocorticoids. Adolescents are the greatest consumers of fructose, and adolescence is a critical period for maturation of the HPA axis. Repeated consumption of high levels of fructose during adolescence has the potential to promote long-term dysregulation of the stress response. Therefore, we determined the extent to which consumption of a diet high in fructose affected behavior, serum corticosterone, and hypothalamic gene expression using a whole-transcriptomics approach. In addition, we examined the potential of a high-fructose diet to interact with exposure to chronic adolescent stress. Male Wistar rats fed the periadolescent high-fructose diet showed increased anxiety-like behavior in the elevated plus maze and depressive-like behavior in the forced swim test in adulthood, irrespective of stress history. Periadolescent fructose-fed rats also exhibited elevated basal corticosterone concentrations relative to their chow-fed peers. These behavioral and hormonal responses to the high-fructose diet did not occur in rats fed fructose during adulthood only. Finally, rats fed the high-fructose diet throughout development underwent

marked hypothalamic transcript expression remodeling, with 966 genes (5.6%) significantly altered and a pronounced enrichment of significantly altered transcripts in several pathways relating to regulation of the HPA axis. Collectively, the data presented herein indicate that diet, specifically one high in fructose, has the potential to alter behavior, HPA axis function, and the hypothalamic transcriptome in male rats.

5.1. Introduction:

Fructose consumption has increased by at least 25% in the past 30 years (Havel, 2005) due to increases in added sweeteners such as sucrose and high-fructose corn syrup. Adolescents are the highest consumers of fructose at 72.8 g/day, with a quarter of adolescents consuming at least 15% of their daily caloric intake from fructose alone (Vos, Kimmons, Gillespie, Welsh, & Blanck, 2008). This is part of a global energy imbalance, resulting in a growing epidemic of metabolic syndrome (Rutledge & Adeli, 2007). The epidemic is not restricted to adults, as today over 20% of American adolescents are obese (Elliott et al., 2002) and Type II diabetes' rates are increasing among youth (Nadeau & Dabelea, 2008).

Diets high in fructose have implications beyond an excess caloric consumption. Such diets alter insulin, blood pressure, and lipid profiles in animal models (Catena et al., 2003; Hwang, Ho, Hoffman, & Reaven, 1987) and humans (Stanhope et al., 2009; Teff et al., 2009). Fructose consumption also raises corticosterone levels in rats (Brindley et al., 1981; Brindley et al., 1985) and elevations in corticosterone may be responsible for fructose-induced hepatic gluconeogenesis (Kinote et al., 2012). The role of glucocorticoids in fructose metabolism is particularly relevant given the clinical data indicating an increased prevalence of depression among diabetic patients (R. J. Anderson et al., 2001). Altered hypothalamic-pituitary-adrenal (HPA) axis signaling is a classic feature of and risk factor for depression (Heim, Newport, Mletzko, Miller, & Nemeroff, 2008).

Chronic stress, which disrupts HPA axis signaling (Bourke et al., 2013) and is associated with increased incidence of depression (Neigh, Gillespie, & Nemeroff, 2009) or depressive-like behavior (Bourke & Neigh, 2011), can exacerbate the effects of diet by promoting palatable food consumption (Pecoraro et al., 2004) and by inducing insulin resistance (Kaufman et al., 2007). A history of early life stress not only increases the risk of depression in adulthood (Bale et al., 2010; Neigh et al., 2009) but also increases the risk of metabolic dysfunction (Williamson, Thompson, Anda, Dietz, & Felitti, 2002). Adolescence is a "critical period" of development that shapes both stress responses (Romeo, 2010) and adult metabolism (Dietz, 1994). For these reasons, we designed our study to examine the interaction of adolescent stress and high-fructose diet on behavior, the HPA axis, and the hypothalamic transcriptome.

We hypothesized that fructose consumption beginning at weaning would induce metabolic disruption paralleling increases in anxiety-like and depressive-like behavior, and that fructose consumption would exacerbate behavioral changes induced by chronic adolescent stress. Further, we hypothesized that these behavioral and metabolic changes would correspond to alterations in HPA axis output both at baseline and in response to an acute stressor. Finally, we used whole-transcriptome RNA sequencing of the hypothalamus to determine the scope of changes in gene expression induced by a high-fructose diet during development.

5.2. Materials and Methods:

5.2.1 Animal Husbandry Timed pregnant Wistar rats (n=22) were obtained on gestational day 12 to produce the periadolescent cohort from Charles River (Wilmington, MA), while male Wistar rats (n=16, PND 56) were obtained from Charles River (Wilmington, MA) to produce the adult cohort. Shipping stress during puberty can alter behavioral outcomes (Laroche et al., 2009b) but shipping of pregnant dams has not been shown to alter developmental outcomes without a pharmacologic challenge (Ogawa, Kuwagata, Hori, & Shioda, 2007a); thus, shipping was conducted during *in utero* development to produce the periadolescent cohort. Animals for

the adult-only diet exposure were obtained from Charles River as adults and acclimated to colony conditions for seven days prior to introduction of the high fructose diet (Capdevila, Giral, Ruiz de la Torre, Russell, & Kramer, 2007). Rats were housed on a 14:10 reverse light:dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). For the periadolescent cohort, litters were culled on postnatal day (PND) 3 to eight pups per litter and weaned on PND 23 (n=134). All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5.2.2 Diet and Metabolic Measurement Either two days post-weaning (Periadolescent Cohort; PND 25; Chow-Non-Stress, n=44; Chow-Stress, n=23; Fructose-Non-Stress, n=43; Fructose-Stress, n=24) or at PND 64 (Adult Cohort, Chow, n=8; Fructose, n=8, all Non-Stress), male rats were pair-housed and assigned to either the Lab Rodent Diet 5001 or a high-fructose diet. The number and usage in each cohort is further clarified in **Supplemental Table 5.1**. In addition, the experimental timelines for each cohort are visualized in **Supplemental Figure 5.1.**

The "periadolescent" cohorts were so named as their diet intake spanned the beginning of adolescence through adulthood, while the "adult" cohort consumed the high-fructose diet during adulthood only. All major outcomes were tested in adulthood for all cohorts. While adolescence is difficult to define precisely in rats as in humans, it is accepted that infancy and "childhood" end at weaning (PND21-23) and that adulthood begins at PND60 (McCormick & Mathews, 2007; Spear, 2000). The diet timelines were thus selected based on the aim to fully cover the adolescent period in the periadolescent cohorts and not in the adult cohorts; and additionally based on evidence from the literature that 8-10 weeks on a similar high-fructose diet will induce metabolic changes (Huang, Chiang, Yao, & Chiang, 2004; Nakagawa et al., 2006).

Non-stressed rats were pair-housed throughout the study, while stressed rats remained pair-housed until the initiation of stress, and single-housed thereafter. The fructose diet used (Research diets D05111802) is 55% fructose while the standard chow (Lab Diet 5001) normally

used is 0.30% fructose. Both diets were supplemented with comparable levels of vitamins and minerals deemed necessary for rodent health, and were reviewed by veterinary staff and approved by IACUC. The details of the macronutrients of each diet have been listed in **Supplemental Table 5.2.** The specifics of the diet used in these studies must also be carefully considered in the context of the observed outcomes. The primary goal of using this diet was to elicit physiologic changes typically associated with fructose consumption in humans, including altered lipid storage and hyperglycemia (Havel, 2005; Tappy & Le, 2010) and to examine concomitant effects on the brain and behavior, and not to mimic common human consumption. This diet is substantially higher than that the average fructose consumption reported in the United States, which is currently estimated at about 10% of total caloric intake for the population on average, and highest among adolescent consumers, with one fourth of adolescents consuming at least 15% of daily calories from fructose (Vos et al., 2008). However, these percentages do not reflect the increase in mass of fructose consumed, as human increased fructose consumption has coincided with substantial increases in caloric consumption, primarily due to increases in carbohydrate consumption (Marriott et al., 2009). In addition, these estimates in humans are based on self-reported dietary recall, which typically underestimates consumption, particularly in obese subjects and adolescents, with obese adults underreporting energy intake by an average 47% and adolescents underreporting by an average 20% (Schoeller, 1995). Nonetheless, it remains clear that the 55% high-fructose diet administered to the rats contains a fructose content substantially higher than that typically consumed. The diet's effects should be understood in the context of an animal model useful for exploring potential effects of a given macronutrient (fructose) on energy homeostasis and stress response, and not as a replica of the human condition.

Metabolic measures were taken from a subset of the periadolescent cohort and the adult cohort. Blood glucose was tested near weekly after an overnight fast by tail prick using a *Freestyle* glucometer. Animal weights were also taken concurrently with glucose readings. Research assistants, carefully accounting for any spilled food, measured food consumption daily

and caloric consumption was determined thereof. To determine caloric efficiency, the body mass gained per week per animal was divided by the mean weekly caloric consumption calculated per cage (of pair-housed animals) divided by two. While imprecise, this type of approximation should only serve to increase variability in caloric efficiency and thus increase probability of returning a false negative result as opposed to producing a false positive.

Fat pads were collected from only a subset of periadolescent animals after weeks on the diet (described below). Weight and fasting blood glucose were assessed in the stress cohorts prior to and after the mixed modality stress, and a subset of the stress & non-stressed animals in each diet cohort were submitted to a glucose tolerance test after eight weeks on the diet. These animals were not used for further behavioral testing.

5.2.3 Fat Pad Collection After nine weeks on either the fructose or chow diets at PND89-90, fructose-fed (n=20) or chow-fed (n=16) animals were either rapidly decapitated (fructose: n=10; chow: n=8) or euthanized and perfused with saline for two minutes (fructose: n=10; chow). Epididymal fat pads were collected from both groups, and peri-renal fat pads were collected from the rapidly decapitated group as described by Casteilla et al (Casteilla, Penicaud, Cousin, & Calise, 2008). Both epididymal and peri-renal fat pads were weighed and normalized to total body weight. In addition, brains were collected and flash frozen on dry ice for later dissection. Trunk blood was collected in EDTA coated tubes and spun at 5400 rcf (3000 rpm in a Stovall SM-24 rotor) for 20 minutes for plasma collection.

5.2.4 Mixed Modality Stress As previously described, a mixed modality chronic stress paradigm that has been shown to elicit behavioral changes in adolescent rats (Bourke & Neigh, 2011; Bourke et al., 2013) was used in this study. Notably, this mixed modality chronic stress paradigm has previously elicited behavioral effects specifically in female rats and not in male rats (Bourke & Neigh, 2011). However, male rats submitted to this chronic stressor showed greater susceptibility to weight gain (Bourke & Neigh, 2011) and an increased neuroinflammatory response to lipopolysaccharide (Pyter et al., 2013) relative to female rats. Thus, this paradigm

and the use of male animals were chosen to maximize the potential to observe an interactive effect of stress and diet without the potential for stress to confound dietary effects.

Animals receiving stress (n=47) were individually housed at PND 35 through the end of the study. These animals were also exposed randomly to either social defeat or restraint for 12 days (PND 37-49). Non-stressed rats remained pair-housed throughout the study (n=67). Social defeat stress was performed during the light phase in the home cage of a mature, territorial, Long-Evans rats for six of the 12 days of defeat. During the social defeat process, an intruder (experimental rat) was placed in the home cage of the resident. After the intruder was attacked by the resident five times on the first day, three times on the second day, and once each day thereafter, or after five minutes, a mesh barrier was placed in the cage, separating the intruder from the resident. This separation continued for 25 minutes. The intruder was then returned to its home cage. The pairings were randomly assigned to prevent stabilization of a dominance hierarchy.

For restraint stress during the remaining six days of stress, animals were placed in a clear acrylic rat restraint (BrainTree Scientific, Braintree, MA, USA) for 60 minutes during the light phase. These restraints prevented head-to-tail turns but did not compress the rat.

5.2.5 Glucose Tolerance Test In order to evaluate the effects of a high-fructose diet initiated post-weaning in conjunction with adolescent stress on glucose metabolism, a subset of rats in each group from the adolescent study were submitted to a glucose challenge. In preparation for this challenge (PND79), a subset of periadolescent chow or fructose-fed stress and non-stress rats were fasted overnight. After weight and blood glucose readings were taken by tail prick using a *Freestyle* glucometer, rats were either given an intraperitoneal glucose bolus (2 g/kg dissolved in saline; n=31) or an equivalent volume of saline (n=29). After one hour, blood glucose was again assessed by tail prick using a *Freestyle* glucometer. Immediately thereafter, rats were rapidly decapitated, trunk blood was collected, and brains were removed and immediately frozen.

5.2.6 Insulin Analysis Insulin was measured in plasma collected from saline- and glucose-treated stressed and non-stressed rats one hour after administration of the saline or glucose bolus, at the conclusion of the glucose tolerance test. Insulin was measured via ELISA (sensitivity 0.1 ng/ml, CrystalChem, Downers Grove, IL). All samples were run in duplicate and those with a CV of <15% were included for analysis.

*5.**2.7 Behavioral Testing*** For the periadolescent cohort (n=8-14 per group), behavioral testing began in adulthood at PND 76, consisting sequentially of open field (PND 76), a 5-minute elevated plus maze test (PND 92), and a 10-minute forced swim test (PND 94). For the adult cohort, behavioral testing began at PND 116 with the same sequence (n=8 per group). The open field test and the swim test were conducted during the middle of the light cycle and the elevated plus maze was conducted two hours after the onset of the dark cycle. All behaviors were recorded by a video camera that was connected to an automated behavior analysis system (CleverSys, Inc, Reston, VA, USA). ***Open field:*** Rats from each group were placed in the center of an open field apparatus that consisted of a square field (75 cm X 75 cm) surrounded by approximately one-meter high plastic walls and allowed to explore for 10 minutes to assess baseline activity. ***Elevated Plus Maze***: An elevated plus maze was used to model anxiety-like behavior in the rats by measuring the time spent in open arms vs the time spent in the closed arms (Pellow, Chopin, File, & Briley, 1985; Walf & Frye, 2007) (n=8-14 per group). The specifications for the San Diego Instruments elevated plus maze were as follows: 43 ½" long, 4" wide (arm width), 19 ½" high (open arms), and 31 ½" high (closed arms). During testing, animals were able to freely move from open to closed arms for 5 min. ***Forced Swim Test:*** The forced swim test has been utilized as a model for depressive-like behavior (Borsini & Meli, 1988; Porsolt, Anton, Blavet, & Jalfre, 1978). While the standard forced swim test uses both pretest and test sessions, we performed only the test session. This single session was used because the original test was developed to assess whether antidepressant drugs administered between the pretest and test day reduced immobility in treated animals (Porsolt et al., 1978). However, when

used to determine whether chronic stress can elicit "behavioral despair" or depressive-like behavior, a single test session has been sufficiently sensitive (Castro et al., 2010; Lutter et al., 2008). In this test, floating was defined as the animal's limbs remaining motionless for at least two seconds, and struggling was defined as the animal's limbs in motion and its head above the surface. Rats were placed in a clear acrylic beaker (40 cm high X 18 cm diameter) filled with room temperature water. Immediately after the end of the 10 min test, rats were removed from the beaker and rapidly decapitated.

5.2.8 Corticosterone Analyses Corticosterone was measured in plasma from blood collected at baseline and immediately after the ten-minute forced swim test in both chow and fructose-fed periadolescent animals via ELISA (sensitivity 27 pg/mL, Enzo Life Sciences, Farmingdale, NY, USA). All samples were run in duplicate.

All blood samples were collected at least two hours before the onset of the animals' dark cycle. For baseline samples, rats were transferred to a testing room two hours prior to the initiation of euthanasia for acclimation. After two hours, rats were rapidly decapitated within two minutes of handling in a different room separated by two doors from the testing room to prevent transfer of scent and noise, and to ensure that decapitation occurred before the rise in plasma corticosterone. For forced swim samples, rats were again transferred to a testing room two hours prior to the initiation of the forced swim test. Subsequently, rats were submitted to the forced swim test for 10 minutes; upon removal from the test, rats were briefly dried then rapidly decapitated within two minutes of removal from the test. Acclimation, swimming, and decapitation all occurred in separate rooms. Upon decapitation, trunk blood was collected in EDTA coated tubes, and brains were removed and immediately frozen. Blood was spun at 5400 rcf (3000 rpm in a Sorvall SM-24 rotor) for 20 minutes for plasma collection for the ELISA.

5.2.9 Whole-Transcriptome RNA-Sequencing Hypothalamic tissue at PND80 was collected from periadolescent fructose and chow-fed rats for evaluation of expression changes. The brains used were from the saline-control-injected, non-stress rats in the glucose tolerance test

as described in **Supplemental Table 5.1**. This cohort was used for RNA-seq analysis to ensure that all rats were in a fasting state, since none of the other cohorts were fasting at the time of euthanasia. This fasting state represents a challenge to energy homeostasis, which has been associated with enhancing neural differences in studies of sucrose and sugar consumption in rodents (Avena, Bocarsly, Rada, Kim, & Hoebel, 2008; Meguid et al., 2000; Minano, Peinado, & Myers, 1989; Myers, Peinado, & Minano, 1988). One brain from the fructose-fed cohort was not included in the RNA-seq for reasons improper handling and storage between removal and RNA extraction thus the total number per group was chow, n=7; fructose, n=6.

Trizol RNA Extraction reagent (Life Technologies) and QiaShredder (Qiagen) were used to lyse and homogenize whole hypothalamus. An RNEasy kit from Qiagen was then used to extract RNA. An Agilent 2100 Bioanalyzer was used to assess RNA purity and quality. One microgram of total RNA was then used to build TruSeq (Illumina) mRNA sequencing libraries. A single end 100 base pair sequencing reaction was performed on an Illumina HiSeq 1000, generating ~25 million reads per sample. The raw sequence reads were mapped to the most recent RAT assembly (RGSC5.0) using the STAR aligner (Dobin et al., 2013). Data fragments per kilobase of exon per million fragments mapped (FPKM) were normalized and differential expression was examined using the Cufflinks software suite (Trapnell et al., 2010). All data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev, & Lash, 2002) and are accessible through GEO Series accession number GSE56238.

5.2.10 Pathway Analysis Pathway analysis of differentially expressed genes established from RNA sequencing was performed using MetaCore (Thomson Reuters, New York City, NY), a web-based computational platform for analysis of high-throughput molecular data (Ekins 2007). The MetaCore database of pathways, networks, diseases, and individual protein-protein, protein-DNA, and protein-RNA interactions is one of the most comprehensive databases, containing over 4.5 million individual findings that are manually annotated based on literature reviews from over 2,700 peer-reviewed journals. Differentially expressed genes discovered through CuffDiff

analysis were uploaded into MetaCore and mapped onto known pathways. Lists of pathways and networks that were significantly enriched with a greater than expected ratio of differentially expressed genes at the FDR < 0.05 were obtained, and specific genes in each pathway were probed for further analysis of function and expression.

5.2.11 Quantitative RT-PCR To assess the effects of the high-fructose diet and chronic adolescent stress on a marker of cerebral metabolism, we examined hippocampal gene expression of glucose transporter-1 (gene name *Slc2a1*; protein name GLUT-1) in periadolescent rats after the several challenges to energy homeostasis, namely, the saline and glucose challenge as well as the forced swim test. Hippocampal RNA was extracted from each animal in the periadolescent cohort after the glucose challenge (saline and glucose) and after the forced swim test (noted in Supplemental Table 5.1).

To evaluate whether the changes in baseline plasma corticosterone could be related to glucocorticoid negative feedback-related gene expression, we examined expression of the following genes: the glucocorticoid receptor (*Nr3cl*)*;* the negative regulator of glucocorticoid signaling, FK binding protein 5 (*Fkbp5*)*;* and the positive regulator of glucocorticoid signaling, peptidyl-prolyl isomerase-D (*Ppid*). These genes were assessed in the non-fasting animals from the metabolic cohort (the same ones used for baseline plasma corticosterone) as a separate validation from the cohort used in RNA-sequencing (noted in Table Supplemental 5.1). These animals were euthanized with Euthasol® after nine weeks on the diet and perfused with saline for two minutes. As previously described, trunk blood was collected, adrenals and testes were collected and weighed, and brains were flash frozen before storage at -80^{oC}. The left hippocampus was then used for RNA extraction.

To validate gene expression changes observed in RNA-sequencing and to determine whether these changes extended to either another brain region or the adult cohort, select targets (pro-opiomelanocortin, *Pomc;* corticotropin releasing factor receptor 1, *Crfr1;* and corticotropin releasing factor receptor 2, *Crfr2)* were evaluated in the hypothalamus and one hemisphere of the

hippocampus for the periadolescent cohort used in RNA-sequencing as well as in these same regions of the adult cohort. As previously described, these animals were rapidly decapitated at either PND80 or PND134, trunk blood was collected, adrenals and testes were collected and weighed, and brains were flash frozen before storage at -80^{oC}. The whole hypothalamus and the left hippocampus was then used for RNA extraction.

For all samples, tissue was lysed and homogenized using Trizol RNA Extraction reagent (Life Technologies, Grand Island, NY) and QiaShredder (Qiagen, Valencia, MA). RNA was extracted with an RNEasy kit from Qiagen, then concentration and purity was assessed with a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). RNA was standardized and then reverse-transcribed using the High Capacity RNA to cDNA kit (Life Technologies, Grand Island, NY). cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized to 10 pg/µl. Rat TaqMan Gene Expression Assays were purchased from Life Technologies (Grand Island, NY) with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively: *Slc2a1* (Rn01417099_m1), *Nr3c1* (Rn00561369_m1); *Fkbp5* (Rn01768371_m1); *Ppid (*Rn01458749_g1); *Pomc* (Rn00595020_m1), *Crfr1* (Rn00578611_m1); *Crfr2* (Rn00575617_m1) and the housekeeping genes *Hprt1* (Rn01527840_m1), *Ubc* (Rn01789812_g1)*, Ppia* (Rn00690933_m1), *Actb* (Rn00667869_m1)*,* and *Tfrc* (Rn01474701_m1). After assessment of suitability, *Hprt1* and *Ppia* were standardized using the geometric mean of the cycle threshold values for the samples from the glucose challenge (calculated as described in (Vandesompele et al., 2002)), while *Hprt1* was used as a housekeeping gene for the samples from the metabolic cohort and *Tfrc* was used as a housekeeping gene for the samples for validation of RNA-sequencing.

The following two-step RT-PCR cycling conditions were used on the 7900HT Sequence Detection System (Applied Biosystems): 50°C (2 min), 95°C (10 min), 40 cycles of 95°C (15 s) and 60°C (1 min). Relative gene expression of individual samples run in triplicate (with coefficient of variation cut-off set to 4%) was determined by the comparative $\Delta\Delta C_T$

quantification method with fold change to standard chow of a given developmental cohort. All TaqMan gene expression assays are guaranteed to have 90 - 100% amplification efficiency as determined by the genome-aided probe and primer design pipeline and reported in the "Amplification Efficiency of TaqMan Gene Expression Assays" Application Note 127AP05-03 from Life Technologies.

5.2.12 Statistical Analysis IBM SPSS Statistics (Version 20), Graphpad Prism (Version 6.0), and RStudio (Version 0.98.1049) were used for statistical analysis and graphing of weight, blood glucose, and food consumption. Unpaired two-tailed Student's t-tests or Analysis of Variance tests were performed with $\alpha=0.05$. Holm-Sidak post-hoc testing was performed when appropriate. For analysis of RNA-seq data, differential expression was set as transcripts in the Fructose dataset that passed False Discovery Rate (FDR) <0.05 as determined using the Cufflinks software suite in R (Version 3.1). This list of transcripts was then imported into the MetaCore^{TM} Analysis Suite (Thomson Reuters, New York City, NY) for pathway analysis. Significant enrichment in pathway map folders and pathway maps was set at FDR<0.05.

5.3. Results:

5.3.1 Periadolescent High-Fructose Diet Increases Caloric Efficiency, Fasting Glucose, and Visceral Fat Pad Mass As determined by repeated-measures ANOVA, rats fed a high-fructose diet beginning at weaning had increased caloric efficiency relative to chow-fed rats, calculated as mg gained per kCal consumed (effect of diet; $F_{1,34}=6.855$, $p=0.0131$; effect of time: $F_{8,272}=488.2$, $p<0.0001$; **Figure 5.1a**), indicating that the fructose-fed rats gained more weight per kCal consumed. Neither the average weekly weight (effect of diet: $F_{1,34}=1.263$, $p=0.2690$) nor the average kCal consumed per week ($F_{1,16}=0.2791$, $p=0.6045$) differed significantly between groups when assessed by repeated measures ANOVA. For both weight and kCal, however, there were significant effects of time (time effect for weight: $F_{9,306}=2998$, $p<0.0001$; time effect for

kCal: $F_{8,128}$=91.05, p<0.0001). Rats fed a high-fructose diet during periadolescence also had elevated fasting glucose beginning only 20 days after initiating the diet (effect of diet: $F_{1,34}$=17.02, p=0.0002; effect of time: $F_{7,238}$=42.80, p<0.0001; **Figure 5.1b**). Raw values for periadolescent weight, kCal, and blood glucose are listed in **Supplemental Tables 5.3, 5.4, and 5.5**. In addition, both peri-renal fat mass (t_{16}=4.270; p=0.0006, **Figure 5.1c**) and epididymal fat mass (t_{34}=3.034; p=0.0046, **Figure 5.1d**) were increased in the periadolescent fructose-fed cohorts.

We also evaluated changes in caloric efficiency and fasting glucose by repeated measures ANOVA in the cohort of rats fed either chow or the fructose diet in adulthood only (PND64-134). Diet had no effect on caloric efficiency (effect of diet: $F_{1,12}$=1.475, p=0.2480, effect of time: $F_{8,96}$=26.61, p<0.0001 **Figure 5.1e**). In addition, neither weight (weight effect: $F_{1,14}$=2026, p=0.6596; time effect: $F_{10,140}$=343.1, p<0.0001) nor kCal (kCal effect: $F_{1,6}$=4.687, p=0.0736; time effect: $F_{7,42}$=4.356, p=0.0010) significantly differed between diets when assessed by repeated measures ANOVA, though there was a trend for the high-fructose fed rats to consume less. Moreover, diet had no effect on fasting blood glucose in the adult cohorts over time (diet effect: $F_{1,14}$=0.6751, p=0.4259; time effect: $F_{9,126}$=12.25, p<0.0001, **Figure 5.1f).** Raw values for adult weight, kCal, and blood glucose are listed in **Supplemental Tables 5.3, 5.4, and 5.5**. Fat pads were not measured in the adult animals.

5.3.2 Periadolescent High-Fructose Diet and Stress Alter Weight Gain but Only Diet Increases Fasting Glucose Though our primary metabolic endpoints were assessed in our non-stress cohorts, we analyzed weight gain in our stress cohorts to confirm the effects of stress and to evaluate potential interaction between stress and diet, as palatable food consumption has been shown to alter stress responses (Dallman et al., 2003). The percent of weight gained during the mid-adolescent stressor (PND37-49) was reduced in the stress cohorts ($F_{1,135}$=28.63, p<0.0001; **Figure 5.2a**) and fructose-fed animals gained more weight during this period ($F_{1,135}$=9.543, p=0.0024; **Figure 5.2a**), though post-hoc testing did not reveal individual significant differences

between groups. The fructose diet also significantly increased fasting glucose during mid-adolescence ($F_{1,131}$=11.37, p=0.0010; **Figure 5.2b)** irrespective of stress history, and post-hoc testing revealed a step-wise increase in change in fasting blood glucose across diet and stress groups. Glucose administration in the glucose tolerance test raised blood glucose in both chow and fructose stress & non-stress groups (main effect of glucose: $F_{(1,24)}$=12.01, p=0.0020) as expected. Fructose diet exacerbated this effect and fructose-fed rats had significantly higher blood glucose in the glucose challenge than the standard chow fed rats ($p<0.05$; **Figure 5.2c**). No effect of stress was detected, nor an interaction with stress observed for either glucose administration or diet ($p>0.40$ for all effects). Only a main effect of glucose administration ($F_{1,42}$=5.0697, p=0.0263, **Figure 5.2d**) was observed in analysis of plasma insulin; neither fructose nor stress, nor an interaction of either with the other or with glucose altered plasma insulin (all $p>0.25$).

5.3.3 Periadolescent High-Fructose Diet Alters Gene Expression of Hippocampal Glucose Transporter-1 after a Glucose Challenge or Acute Stress We examined hippocampal gene expression of glucose transporter-1 (*Slc2a1*) as a possible marker of changes in cerebral metabolism. Data were first analyzed among the non-stressed animals to assess the effects of the glucose challenge and forced swim test at baseline (**Figure 5.3a,b**). There was a significant effect of diet on *Slc2a1* expression (**Figure 5.3b,** $F_{1,24}$=10.31, p=0.0037) and an interaction with acute stress (forced swim; $F_{1,24}$=12.41, p=0.0017) in non-stressed animals at baseline. Post-hoc testing indicated that these effects were due to a significant increase in *Slc2a1* expression after forced swim in chow-fed animals only. In the glucose challenge, *Slc2a1* expression was not altered by glucose administration (**Figure 5.3a,** $F_{1,23}$=1.127, p=0.2994), diet ($F_{1,23}$=2.884, p=0.1030), or an interaction of glucose and diet ($F_{1,23}$=2.321, p=0.1412) in non-stressed animals.

When including the chronically stressed animals, the high-fructose diet significantly reduced hippocampal *Slc2a1* expression after both the glucose challenge (**Figure 5.3d,** $F_{1,24}$=4.691, p=0.0405) and after the forced swim test (**Figure 5.3e,** $F_{1,26}$=18.81, p=0.0002), without an effect of stress. However, there was no effect of diet (**Figure 5.3c,** $F_{1,23}$=1.178, p=0.2889), stress ($F_{1,23}$=0.2812,

p=0.6010), or an interaction of stress and diet ($F_{1,23}$=1.845, p=0.1875) after saline alone. In the glucose challenge, there was no significant effect of either stress or an interaction of stress and diet (both p>0.65), nor were there any significant post-hoc effects. In the forced swim test, there was no significant effect of either stress or an interaction of stress and diet (both p>0.15), but post-hoc testing indicated that both non-stress and stressed fructose animals had significantly reduced *Slc2a1* expression relative to chow-non-stress, but not relative to chow-stress.

5.3.4 Periadolescent high-fructose feeding does not alter gene expression of glucocorticoid signaling factors Periadolescent fructose-fed animals did not differ from chow-fed animals in expression of the glucocorticoid receptor (*Nr3c1,* $t_{10.21}$=0.4511, p=0.6265), FK binding protein-5 (*FKBP5*, t_{13}=0.0008, p=0.9994), or peptidyl-prolyl isomerase-d (*Ppid,* $t_{10.93}$=0.3591, p=0.7264; data not shown).

5.3.5 Periadolescent High-Fructose Diet Increases Anxiety-like Behaviors. Rats that began consumption of the high-fructose diet at weaning showed elevated levels of anxiety-like behaviors in adulthood after 8-10 weeks on the diet (n=8-14 per diet & stress group). Anxiety-like behavior was assessed using the open field (Prut & Belzung, 2003) and elevated plus maze (Pellow et al., 1985). In the open field, periadolescent fructose-fed rats traveled farther ($F_{1,34}$=4.814, p=0.0352, **Figure 5.4a)** and faster ($F_{1,34}$=5.083, p=0.0307, data not shown) than the standard chow-fed rats in the open field test. Stress did not affect either of these measures (p>0.05 for all effects) nor was there an interaction between stress and diet (p>0.05 for all effects). Diet ($F_{1,34}$=10.86, p=0.0023; **Figure 5.4b**) and stress ($F_{1,34}$=5.241, p=0.00284) both independently affected central tendency, and also interacted to affect central tendency ($F_{1,34}$=8.974, p=0.0051). Post-hoc testing indicated that the stress effect was due to an increase in central tendency in the chow-fed stressed cohort (p=0.0017 versus non-stressed chow-fed cohort).

Consistent with an anxiogenic phenotype, rats fed a high-fructose diet throughout adolescence spent less percent time in the open arms of the elevated plus maze than standard chow-fed rats ($F_{1,34}$=9.838, p=0.0035, **Figure 5.4c).** Chronic stress neither independently altered

percent time in the open arms nor interacted with the high-fructose diet to modify behavior ($p>0.05$ for both). However, when assessing entries into the open arm, both stress and diet altered open arm entries, with stress increasing open arm entries ($F_{1,34}=10.34$, $p=0.0029$; data not shown) and diet reducing open arm entries ($F_{1,34}=8.225$, $p=0.0070$), but without an interaction effect ($p>0.90$).

High-fructose diet consumption in adulthood only did not alter either locomotor activity ($t_{14}=1.183$, $p=0.2567$, **Figure 5.4d**) or central tendency ($t_{14}=0.2562$, $p=0.8015$, **Figure 5.4e**) in the open field. In the elevated plus maze, rats that started the fructose diet in adulthood also behaved similarly to chow-fed rats (open arm percent time: $t_{14}=0.7009$, $p=0.4948$, open arm entries: $t_{14}=0.8303$, $p=0.4203$; **Figure 5.4f).**

5.3.6 Periadolescent High-Fructose Diet Increases Depressive-like Behaviors Rats that began consumption of the high-fructose diet at weaning showed elevated levels of anxiety-like behaviors in adulthood after 8-10 weeks on the diet (n=8-14 per diet & stress group). This change was prominently observed in the forced swim test, a test of depressive-like behavior in rats (Porsolt, Le Pichon, & Jalfre, 1977). In the forced swim test, fructose-fed rats spent less time struggling than standard chow fed rats ($F_{1,22}=4.501$, $p=0.0165$; **Figure 5.5a)** and had reduced latency to float ($F_{1,23}=6.825$, $p=0.0156$; see Figure **5.5b**). Further, the fructose-fed rats spent more time immobile in the forced swim test ($F_{1,22}=6.745$, $p=0.0454$, **Figure 5.5c)**. Contrary to our original hypothesis, stress did not independently influence behavior nor did it interact with diet in the forced swim test in any measure regardless of diet ($p>0.05$ for all measures). The reduced activity in the forced swim test could not be attributed to reduced motor activity because, as previously described, fructose-fed rats traveled farther ($F_{1,34}=4.814$, $p=0.0352$, **Figure 5.4a)** and faster ($F_{1,34}=5.083$, $p=0.0307$, data not shown) than the standard chow-fed rats in the open field test.

Rats fed the high fructose diet during adulthood only did not change their behavior relative to their chow-fed controls in either struggling ($t_{14}=0.2769$, $p=0.7859$, **Figure 5.5d**) or

immobility (t_{14}=0.7054, p=0.4921, **Figure 5.5e**) in the forced swim. However, there was a trend toward reduced latency to float in the adult fructose-fed rats (t_{14}=2.094, p=0.0549, **Figure 5.5f).**

5.3.7 Periadolescent High-Fructose Diet Interacts with Acute Stress to Alter Plasma Corticosterone Previous reports have indicated that fructose is sufficient to alter HPA axis activity (Kinote et al., 2012). Additionally, we have demonstrated that rats exposed to the high-fructose diet beginning at weaning demonstrated elevated depressive-like and anxiety-like behaviors (**Figures 5.4 and 5.5**), which is consistent with a robust literature on the relationship between HPA axis activity and affective behavior (Heim et al., 2008). Therefore, we evaluated basal and acute stress-induced HPA axis output through examination of terminal plasma corticosterone concentrations. Forced swim elevated plasma corticosterone as expected ($F_{1,40}$-239.5; p<0.0001) and diet interacted with forced swim to affect plasma corticosterone ($F_{1,40}$-7.042; p=0.01; **Figure 5.6a**). This interaction could be explained by a significant elevation of plasma corticosterone at baseline in the fructose-fed animals (mean difference 114.1 ng/ml, p<0.05), but there existed either no significant difference or a slight trend toward a reduction in plasma corticosterone in the fructose-fed animals after the FST (mean difference -101.9 ng/ml, p=0.12).

To determine whether the effects of fructose on plasma corticosterone were specific to developmental exposure, we examined the basal corticosterone concentrations in rats fed the high-fructose diet or chow in adulthood only. No effects of high-fructose diet consumption in the adult cohort were observed (t_{14}=1.065, p=0.3048; **Figure 5.6b**).

5.3.8 Periadolescent High-Fructose Diet Remodels the Hypothalamic Transcriptome with Greatest Impact on POMC Processing. Given the robust effects of developmental fructose diet on behavior (**Figures 5.4 and 5.5**), the HPA axis (**Figure 5.6**), and metrics of metabolism (**Figures 5.1 and 5.2**), we examined the consequences of a periadolescent high-fructose diet on the hypothalamic transcriptome. Hypothalamic tissue at PND80 was collected from periadolescent fructose and standard rats for evaluation of expression changes. RNA was

extracted and used for whole-transcriptome RNA sequencing. Of the 17,366 transcripts assessed, 966 or 5.56% were significantly differently expressed between the standard chow cohort and the fructose chow cohort, after correction for the false discovery rate (**Figure 5.7**). Analysis was performed using the MetaCore™ Analysis Suite (Thomson Reuters, New York City, NY) to determine pathways and networks that were significantly enriched with differentially expressed genes. Pathway analysis revealed significant enrichment of 97 pathways with differentially expressed genes (all $q<0.05$; **Supplementary Table 5.6**, top 25 pathways). Notably, multiple pathways relating to HPA axis function, including "POMC Processing" (the pathway with the highest ratio of significantly altered transcripts), "Post-translational processing of neuroendocrine peptides," and "Corticoliberin signaling via CRFR1" were significantly enriched with differentially expressed genes (all $q<0.001$). Network analysis, which can link multiple pathways through interacting genes, demonstrated significant enrichment of 25 networks with differentially expressed genes (all $q<0.05$, **Supplementary Table 5.7**), with the top ranking networks pertaining to synaptic contact and neuropeptide signaling. Significantly differentially expressed genes in the CRF receptor and POMC processing pathways are shown in **Figure 5.8** by denotation with an asterisk**.**

5.3.9 High-Fructose Diet Upregulates Hypothalamic Expression of *Pomc* and *Crfr1* when Consumed during Periadolescence but Only *Pomc* when Consumed during Adulthood

Quantitative RT-PCR was used to validate three genes, *Pomc, Crfr1, and Crfr2,* that were identified in RNA-sequencing as potentially altered due to periadolescent high-fructose diet. As shown in Figure 6b, hypothalamic *Pomc* and *Crfr2* were significantly upregulated in the RNA-sequencing dataset, while *Crfr1* was non-significantly downregulated. Consistent with these findings, RT-PCR indicated upregulation of hypothalamic *Crfr2* ($t_{11}=2.978$; $p=0.0126$, **Figure 5.9c**) and a strong trend toward upregulation of *Pomc* ($t_{11}=2.192$; $p=0.0508$, **Figure 5.9a**) in periadolescent fructose-fed animals without an effect on *Crfr1* ($t_{11}=0.3224$; $p=0.7532$, **Figure 5.9b**). In animals fed fructose during adulthood only, there was a trend indicating upregulation of

Pomc (t_{10}=2.087; p=0.0635, **Figure 5.9d**) but no effect was observed on either *Crfr1* (t_9=0.8502; p=0.4173, **Figure 5.9e**) or *Crfr2* (t_4=0.0732; p=0.9452, **Figure 5.9f**). In addition, fructose consumption had no effect on hippocampal *Pomc, Crfr1,* or *Crfr2* in either the periadolescent or the adult cohort (all p>0.10, data not shown).

5.4. Discussion:

Consumption of a diet high in fructose altered behavior, physiology, and gene expression, irrespective of the rats' stress histories, yet dependent on the developmental stage during which the diet was consumed. Only rats fed the high-fructose diet beginning at weaning showed increased anxiety-like and depressive-like behaviors in adulthood; rats that began the diet after reaching adulthood did not manifest the same behaviors despite the same total exposure to the diet. Moreover, rats fed fructose since weaning had elevated basal corticosterone relative to chow-fed rats. Surprisingly, the combination of the high-fructose diet with exposure to chronic adolescent stress did not produce any additional effects. Finally, the periadolescent high-fructose diet induced remodeling of the hypothalamic transcriptome, with 966 transcripts significantly altered and a dramatic enrichment of altered transcripts in the POMC pathway.

Chronic periadolescent fructose exposure induced both anxiety-like and depressive-like behaviors when assessed in adulthood. Periadolescent fructose-fed rats traveled less in the open arms of the elevated plus maze (**Figure 5.4c**), a validated test of anxiety-like behavior in the rat (Pellow et al., 1985). Similarly, diet reduced central tendency (**Figure 5.4b**), another metric of anxiety (Prut & Belzung, 2003) in the animals exposed during periadolescence. It should be noted that in these metrics, stress increased open arm entries in both chow- and fructose-fed animals and increased central tendency in chow-fed animals only. These increases in exploratory behavior, specifically in the open field and elevated plus maze, have been previously observed in animals stressed during adolescence (Jacobson-Pick & Richter-Levin, 2010; Toledo-Rodriguez & Sandi, 2011), though the effects in adulthood may be reversed (Jacobson-Pick & Richter-Levin,

2010). In the present study, the high-fructose diet blunted the exploratory effects of the adolescent stress paradigm used and instead induced an anxiogenic phenotype.

In the forced swim test, a validated test of depressive-like behavior (Porsolt et al., 1977), periadolescent fructose-fed rats struggled less, exhibited a decreased latency to float, and increased floating time in the forced swim test, consistent with a depressive-like phenotype (**Figure 5.5).** Adolescent stress had no effect on any of these metrics. In addition, the increased immobility was observed alongside increased activity in the open field, demonstrating that consumption of a high-fructose diet did not cause a global reduction in motor behavior (**Figure 5.4a**). Moreover, hyperactivity in the open field has been linked to anxiety-like behavior (Ito, Nagano, Suzuki, & Murakoshi, 2010), consistent with the reductions in open arm entries and central tendency.

As the animals that consumed fructose during adulthood only had no difference in elevated plus maze behavior or immobility and struggle behavior in the forced swim test **(Figures 5.4 and 5.5)**, the effects on anxiety- and depressive-like behavior induced by the high-fructose diet appeared to be developmentally specific. It is notable that there was a trend toward reduced latency to float in the adult animals, and given the use of only eight animals per group, results may be underpowered to detect significant differences at the 0.05 level. Indeed, power analysis in each behavioral test indicated power of less than 0.40 in each test. Thus, the data presented here cannot rule out all behavioral effects that a high-fructose diet may have in adulthood. However, the effect size on behavioral outcomes in the adult animals is generally less than in the periadolescent animals, giving rise to the hypothesis that the adult animals may be less susceptible to the behavioral influence of the diet.

In terms of metabolic abnormalities, rats fed the high-fructose diet during adolescent development also appeared uniquely susceptible relative to rats fed the diet during adulthood only. While periadolescent fructose-fed rats had increased caloric efficiency and increased blood glucose over the course of the experiment, rats fed fructose in adulthood only experienced no

changes in caloric efficiency or blood glucose (**Figure 5.1**). For caloric efficiency, however, it should be noted that the method of calculation introduced variability and the lack of effect in the adult cohort could be indicative of a false positive. However, the difference between the periadolescent fructose and chow fed animals is notable in spite of this high variability. Periadolescent rats also had increased adiposity, though this effect was not tested in adult animals. Interestingly, though the effects of stress on behavior were not significant, both stress and diet affected energy homeostasis. During the stress period, stress reduced weight gain, while the fructose diet promoted increased weight gain (both main effects; **Figure 5.2**). Though only fructose had a significant effect on blood glucose both during the stress period (**Figure 5.2b**) and during the glucose tolerance test (**Figure 5.2c**), it is notable that the combined effects of stress and fructose induced a step-like pattern to increase blood glucose during the stress period. The single time point in the glucose tolerance test is a limitation of this study, as we cannot determine if changes in blood glucose in the fructose-fed rats are due to a prolonged return to baseline or an elevated peak. Other studies have done such a time-course study using in male rats fed a high-fructose diet (60-66% fructose) for two (Catena et al., 2003), eight (Huang et al., 2004), or ten weeks (Nakagawa et al., 2006) in adulthood only. Similar to the present study, none of these experiments observed baseline differences in fasting blood glucose in the animals fed in adulthood; however, after two weeks on the diet, differences in blood glucose were observed at 15 and 30 minutes after administration of a glucose bolus (Catena et al., 2003). After eight weeks on the diet, blood glucose differed between chow and fructose-fed rats at 120 minutes after glucose administration; but after ten weeks on the diet, blood glucose was equivalent at all time points between the two diet groups . All of these studies, however, observed significant differences in plasma insulin by the thirty-minute time point (Catena et al., 2003; Huang et al., 2004; Nakagawa et al., 2006). In the present study, however, no differences in plasma insulin were observed indicating that insulin resistance had not developed at the time point examined.

Future studies will be necessary to determine the dynamics of the response in periadolescent stressed and non-stressed animals and to further assess the dynamics in adult animals.

The periadolescent diet not only influenced peripheral metabolism (**Figures 5.1 & 5.2**), but it also altered gene expression of the cerebral metabolic marker, GLUT1. As discussed in Chapters Two and Three, cerebral glucose transporters facilitate glucose transport across the blood-brain-barrier as well as the uptake of glucose into neurons and glia (Brown, 2000; B. S. McEwen & Reagan, 2004; Simpson et al., 2007). Disruptions in glucose transporter expression or activity are linked to multiple neuropathologies including Alzheimer's disease, De Vivo's disease, and traumatic brain injury (Cornford et al., 1996; Hamlin et al., 2001; Simpson, Chundu, et al., 1994; Vannucci, Reinhart, et al., 1998; Vannucci et al., 1996). We have previously shown in Chapter Two that exposure to either adolescent or adult chronic stress increases hippocampal *Slc2a* in male but not female rats. Here, exposure to an acute stressor (the ten-minute forced swim test) was sufficient to increase hippocampal *Slc2a* (**Figure 5.3b**); however, periadolescent fructose-fed rats did not mount this same increase in *Slc2a* in response to acute stress. Stressed and non-stressed fructose-fed rats had reduced *Slc2a* expression after both the glucose challenge (**Figure 5.3d**) and after the forced swim test (**Figure 5.3e**), potentially indicating an impaired ability to respond to the increased energetic challenge. Interestingly, unlike their non-stressed counterparts (**Figures 5.3a,b**), the stressed chow-fed animals also did not increase expression of *Slc2a1* after either glucose administration or the forced swim test (**Figures 5.3d,e**). However, they started from a higher basal level of expression, consistent with Chapter Two (**Figure 5.3c),** and may reached a "ceiling effect."

Unlike rats fed in adulthood only, periadolescent fructose-fed rats also had elevated baseline plasma corticosterone relative to the standard chow-fed rats, irrespective of stress history (**Figure 5.6**) but consistent with earlier studies showing that acute fructose consumption raises corticosterone levels in rats (Brindley et al., 1981; Brindley et al., 1985). Brindley et al (Brindley et al., 1981) observed that rats fed a high starch diet in particular had an elevated peak

corticosterone response to acute fructose as well as a prolonged return to baseline, though this trend was evident across diets relative to acute glucose or acute sodium feeding. Though differences in the response to acute stress were not observed in the present study, it is possible a time course study after acute stress would elicit more information. For example, rats fed a high-fat diet for nine-weeks had a higher integrated area under the curve of the plasma corticosterone response across 120 minutes following acute stress (Tannenbaum et al., 1997). Palatable food consumption in the short term has been hypothesized to blunt HPA axis output and the stress response (Pecoraro et al., 2004); however, over the long term, diet-induced shifts in energy homeostasis can alter this effect. Dietary macronutrients are known to affect peripheral glucocorticoid metabolism, such that higher fat/lower carbohydrate diets can alter hepatic and adipose 11β hydroxysteroid dehydrogenase (11βHSD) as well as 5α- and 5β-reductase (Stimson et al., 2010). Similar changes in glucocorticoid metabolism have been observed in the context of obesity, with notably lower hepatic 11βHSD in obese patients resulting in impaired activation of cortisone to cortisol in the liver (Stewart, Boulton, Kumar, Clark, & Shackleton, 1999) and potentially leading to an increased drive of the HPA axis (Rask et al., 2001). In addition, changes in corticosterone output have frequently been observed in chronic stress models (Plotsky & Meaney, 1993) and in adolescent stress models specifically (Bourke et al., 2013; McCormick et al., 2011). Though alterations in the hippocampal glucocorticoid receptor and its co-chaperones have been implicated in altered corticosterone signaling after adolescent stress (Bourke et al., 2013), stress- or diet-induced alterations on these factors are less likely to play a role in the present study, since they were unaffected in PCR analysis. Such dysfunctional HPA axis activity has similarly been linked to clinical mood disorders (Heim et al., 2008).

It is possible that the fact that the periadolescent animals were more vulnerable to the effects of the diet due to the fact that the diet was consumed during a "critical period" of development. This concept of "critical periods" of development has been extensively examined across multiple developmental periods, including both prenatal (McCormick, Smythe, Sharma, &

Meaney, 1995; Pankevich et al., 2009; Tamashiro et al., 2009) and adolescent (Bourke & Neigh, 2011; McCormick & Mathews, 2007) periods. Adolescence in the rat typically encompasses PND28-55 and consists of numerous hormonal, behavioral, and neural changes (Spear, 2000). Hormonal changes that occur during adolescence include adrenarche, or the increase in output of adrenal hormones, and gonadarche, the pubertal increase in output of gonadal hormones (Spear, 2000). This awakening of hypothalamic output coincides with neural changes, including sexually dimorphic evolution of the locus coeruleus as well as expansion and contraction of striatal and prefrontal dopamine receptors (Sisk & Zehr, 2005).

Adolescence is also a challenge period for energy homeostasis; puberty, one of the transitions comprising adolescence, is highly linked to metabolic changes (Spear, 2000). The onset of puberty more highly linked to body weight and food intake than chronological age (Kennedy & Mitra, 1963), likely mediated by neuroendocrine factors such as leptin (Mantzoros, Flier, & Rogol, 1997). Perhaps because of this relationship, adolescence has been termed a critical period for obesity in particular (Alberga, Sigal, Goldfield, Prud'homme, & Kenny, 2012; Dietz, 1994, 1997). For example, overweight and obese adolescents (BMI >75th percentile) in the 1922-1935 Harvard Growth Study had relative risks of 1.8 (confidence interval (CI) 1.2-2.7) for all-cause mortality and of 2.3 for coronary artery disease 55 years later, even when controlling for weight at age 53, relative to adolescents with a BMI between the 25th and 50th percentile (Must, Jacques, Dallal, Bajema, & Dietz, 1992). Adolescence thus appears to be a critical period for hormonal, behavioral, neural, and metabolic outcomes. In the present study, the dietary manipulation extended beyond the period of adolescence, hence its consideration as a periadolescent diet. The precise times during periadolescence encompassing the critical period for the dietary effects to take place cannot yet be determined. However, the evidence remains that rats that consumed the diet during this period as opposed to during adulthood only were more susceptible to the metabolic, hormonal, behavioral, and molecular changes. Future studies, for example examining potential effects of a shorter diet duration confined to adolescence and

weaning onto adult chow, might enable better characterization of the precise timing of this metabolic critical period.

In the present study, periadolescent high-fructose diet, though damaging on metabolic and behavioral outcomes, did not exacerbate the effects of the chronic adolescent stress exposure. Consistent with previous reports on the effects of chronic mixed modality stress in adolescent male rats (Bourke & Neigh, 2011), this chronic adolescent stress paradigm had only minor effects on behavior. This particular stressor was selected to facilitate observation of the additive effects of diet and stress, and to determine whether an additional "hit" in the form of diet could precipitate stress effects on behavior not previously seen. While depressive- and anxiety-like behavioral outcomes of stress were not exacerbated by diet in this study, we cannot rule out that using a different stressor or examining different behavioral tests would have yielded different results, as some adolescent stressors have shown lasting behavioral effects into adulthood in males (McCormick et al., 2012). Further, given that the current study employed only males, we cannot rule out that an interaction might occur in females who are more susceptible to behavioral effects of adolescent stress (Bourke & Neigh, 2011; McCormick, Smith, & Mathews, 2008). The developmental upbringing of the rats must also be considered as a potential factor affecting the differences between cohorts; the periadolescent rats were born and raised in-house, while the adult animals were raised at a vendor's facility. In particular, the relative litter size could affect later appetite and feeding behavior; both early life undernutrition and overnutrition have been associated with adulthood obesity (reviewed in (Langley-Evans, Bellinger, & McMullen, 2005; Patel, Srinivasan, & Laychock, 2009)). However, the timing of shipping was selected so as not to alter outcomes (Capdevila et al., 2007; Ogawa et al., 2007a). In addition, given that the current study employed only males, we cannot rule out that an interaction or additive effect of stress might occur in female rats, which are more susceptible to behavioral effects of adolescent stress (Bourke & Neigh, 2011; McCormick et al., 2008). Nonetheless, the data presented indicate that a periadolescent high-fructose diet has a greater impact on male rat behavior than chronic

adolescent stress, and that the dietary impact on behavior may be specific to the developmental period of exposure.

Adding to these behavioral and physiological changes, the periadolescent high-fructose diet also reprogrammed hypothalamic transcript expression, affecting over 5% of all transcripts, with the POMC pathway and other pathways relating to processing of neuroendocrine peptides particularly affected (**Figures 5.7 and 5.8; Supplementary Tables 5.6 and 5.7**). POMC is a precursor polypeptide primarily produced in the hypothalamic arcuate nucleus as well as corticotrophs and melanotrophs anterior pituitary that is cleaved enzymatically into multiple downstream hormones. Cleavage of POMC occurs in a cell-type-specific manner; for example, in pituitary corticotrophs, POMC is cleaved into neurotensin (NT), joining peptide, ACTH, β – lipotropin (βLPH), and γLPH, and β-endorphin, whereas in hypothalamic melanotrophs, POMC is further cleaved into γ-melanocyte-stimulating hormone (γMSH)s from NT, to αMSH and corticotrophin-like intermediate lobe peptide from ACTH, and βMSH from βLPH as well as β endorphin (Raffin-Sanson 2003). Adipose tissue-derived leptin, insulin, and potentially glucocorticoids act on POMC neurons in the arcuate nucleus to promote POMC expression (Cowley et al., 2001) and stimulate POMC neurons to stimulate melanocortin-4 or 3-receptors in the PVN via αMSH and cocaine and amphetamine related transcript (Coll 2004; Ramamoorthy 2015). POMC neurons additionally maintain a reciprocal relationship with Agrp/NPY neurons in the arcuate nucleus, which act in contrast to POMC to stimulate feeding (Hahn 1998).

In the present study, RNAseq analysis as well as PCR validation indicated that *Pomc* was upregulated in the hypothalamus of both periadolescent and adult fructose-fed animals (**Figures 5.5 & 5.6**). This is consistent with evidence from high-fat diet studies in mice indicating that exposure to a high-fat diet induces upregulation in *Pomc* as a potential defense against obesity (Ziotopoulou, Mantzoros, Hileman, & Flier, 2000), and indicates potential susceptibility to hypothalamic remodeling in both the periadolescent and adult animals. Remodeling appeared to be more extensive in the periadolescent animals, as RNAseq analysis and PCR validation

revealed that C*rfr2* was upregulated in the periadolescent high-fructose cohort but not the adult high-fructose cohort. In response to stress, secreted CRF stimulates CRFR1, which has a higher affinity for CRF than CRFR2 (Charmandari et al., 2005) and is traditionally associated with depressive-like and anxiety-like behavior (reviewed in (Bale & Vale, 2004)). Consistent with this finding that periadolescent fructose consumption alters CRFR2, the urocortins, which activate CRFR2 and may be involved in modulating the stress response (Bale & Vale, 2004; Charmandari et al., 2005), were also altered in RNAseq analysis (**Figure 5.8**). While deficiency in CRFR2 is associated with increased stress sensitivity (Bale et al., 2000), upregulation in the periadolescent fructose-fed animals may be a compensatory mechanism to combat the increases in baseline corticosterone, depressive- and anxiety-like susceptibility, and divergence from the HPA axis' homeostatic norm. It is possible that increases in *Pomc* expression in both periadolescent and adult animals indicate hypothalamic remodeling to counteract the metabolic effects of the diet, while only the periadolescent animals demonstrate remodeling to counteract the anxiogenic effects of the diet. However, both of these changes may underlie behavioral, metabolic, and hormonal outcomes seen in the fructose-fed rats.

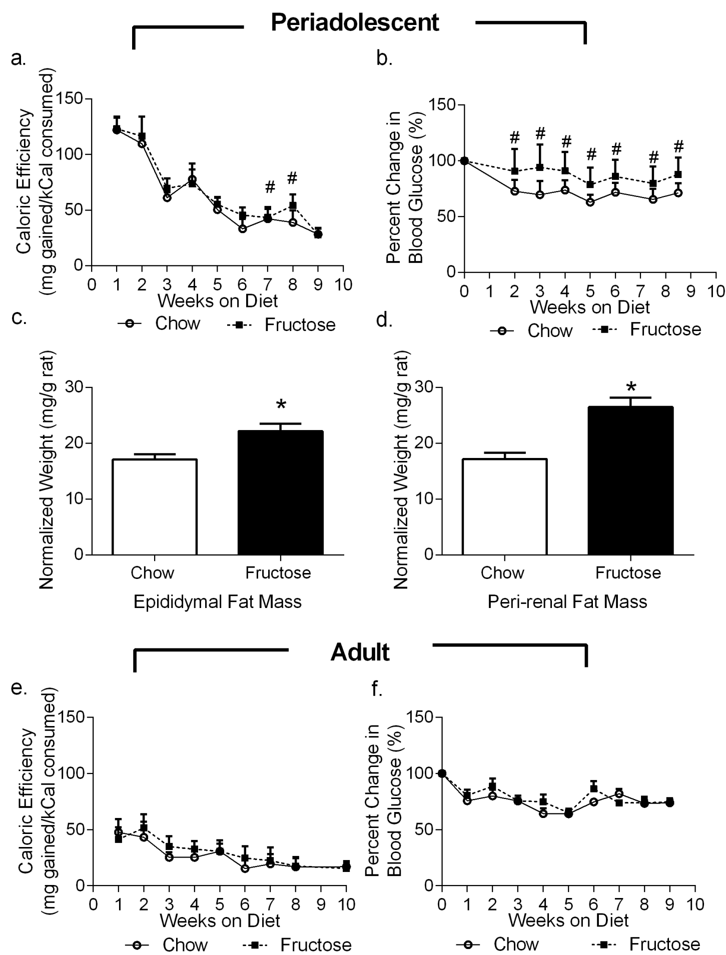
Taken together, the results in this study indicate that consumption of a high-fructose diet from weaning throughout adolescent development and into adulthood is sufficient to alter metabolic indices, increase basal plasma corticosterone, induce anxiety- and depressive-like behaviors, and promote widespread changes in the hypothalamic transcriptome, particularly affecting genes and pathways related to stress and feeding. These results are consistent with the literature indicating that disruptions to energy homeostasis, such as in states like obesity and diabetes, are associated with depressive-like behavior and altered HPA axis function. A recent meta-analysis confirmed the relationship between depression and overweight or obesity, focusing on longitudinal analyses to examine the reciprocal link between the two disorders (Luppino et al., 2010). Obesity is not the only metabolic disorder associated with depression, as the prevalence of depression is doubled in diabetics (R. J. Anderson et al., 2001) and depression increases the risk

of diabetes (Eaton, Armenian, Gallo, Pratt, & Ford, 1996; Knol et al., 2006). Metabolic disorders such as diabetes may be capable of acting as stressors on multiple levels to mediate a functional change in the HPA axis and induce susceptibility to depression (Musselman & Nemeroff, 1996; Reagan, 2012). For example, low-grade inflammation (Lamers et al., 2013), alterations in leptin or neuropeptide Y (Heilig, Soderpalm, Engel, & Widerlov, 1989; Sajdyk et al., 2008), and changes in the gut microbiome (Bailey et al., 2011) are just a few of the possible mechanisms whereby shifts in energy homeostasis have been observed to affect behavior and the stress response.

Future research will be necessary to disentangle the effects of the metabolic effects of fructose from any potential direct effects of this monosaccharide on neural and hormonal function. Pair-feeding could be one way to address this issue that would shed light on the effect of food intake and shifts in basal metabolism on outcome. However, in light of the reduced consumption yet equivalent weight gain (consistent with the increased caloric efficiency) in the periadolescent animals, “pair-starving” the chow group, or force-feeding the fructose group, might only exacerbate the effects. Alternative methods to address this confound between the direct versus indirect of fructose could be to induce increased adiposity or hyperglycemia through other means, potentially by comparing diets (i.e., introduction of a high-fat diet); or by pharmacologically inducing hyperglycemia or genetically inducing obesity. Continuing studies should also seek to determine whether the effects of the periadolescent high-fructose diet are reversible. The literature regarding reversibility of diet or metabolic dysfunction after consumption during a critical stage is mixed. For example, maternal high-fat diet can induce epigenetic changes associated with changes in body size and insulin sensitivity lasting up to two generations (Dunn & Bale, 2009). However, weaning onto a chow diet after exposure to a maternal high-fat diet reverses body mass differences in non-stressed rats (Tamashiro et al., 2009). The persistence of effects after consumption during adolescent development remains to be determined.

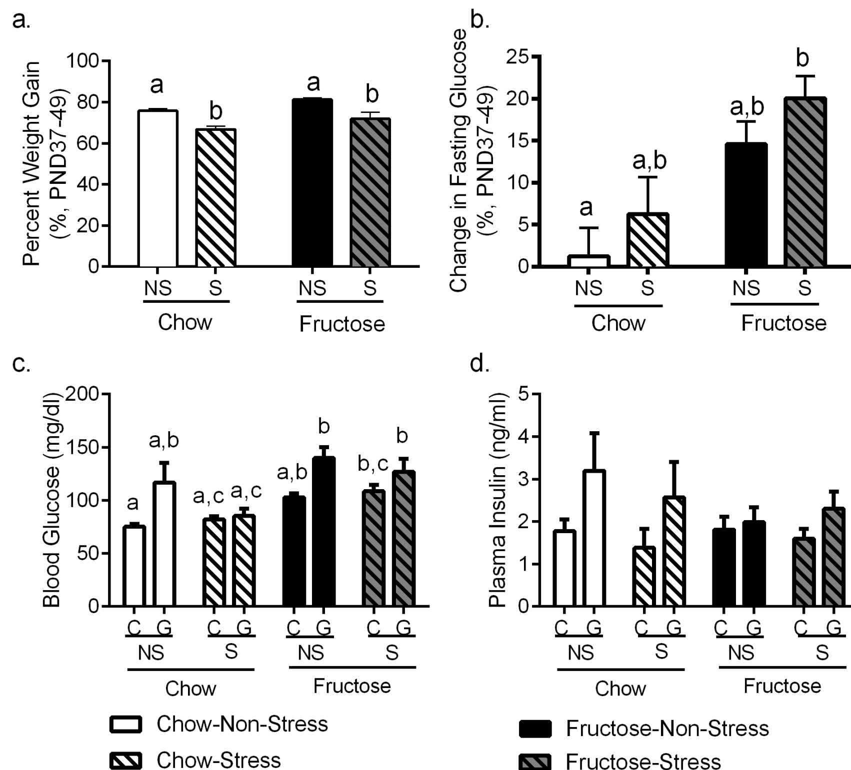
In conclusion, we expand on prior studies that have examined stress responses in diet models to demonstrate effects of high-fructose diet and stress on behavior, physiology, and gene expression. This study indicates that a high-fructose diet promotes depressive- and anxiety-like behavior independent of chronic adolescent stress in male rats. The data corroborate the importance of the adolescent critical period, as the effects of the high-fructose diet were evident only when consumed throughout adolescence. We also demonstrate a substantial shift (more than 5% of genes affected) in hypothalamic gene expression after consumption of a high-fructose diet during adolescence, most notably in genes and pathways regulating stress and feeding. Collectively, the data presented herein indicate a strong potential for diet, specifically high-fructose consumption, to alter behavior and reprogram the HPA axis both in terms of function and at the level of the transcriptome in the male rat.

Figure 5.1.



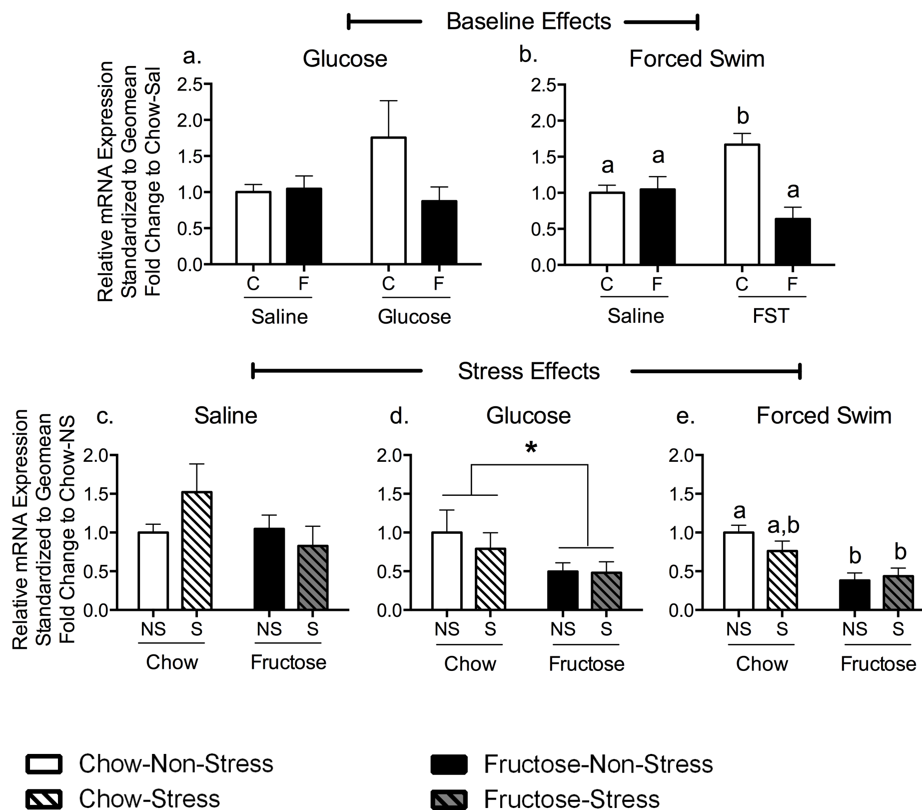
Periadolescent High-Fructose Diet Alters Metabolic Parameters a. Rats fed a high-fructose diet during periadolescence had increased caloric efficiency relative to chow-fed rats. b**.** Periadolescent fructose-fed rats had significantly higher fasting blood glucose relative to chow-fed rats beginning within 20 days on the diet. c, d. Both epididymal and retroperitoneal fat mass were increased in the periadolescent fructose-fed rats relative to their chow-fed controls. e,f. In rats fed the high-fructose diet during adulthood only, neither caloric efficiency nor blood glucose significantly differed from chow-fed rats. Data shown are mean ± SEM; asterisk indicates an effect of diet in the t-test and a hashtag indicates a significant post-hoc effect between diets at the same timepoint with $p < 0.05$.

Figure 5.2.



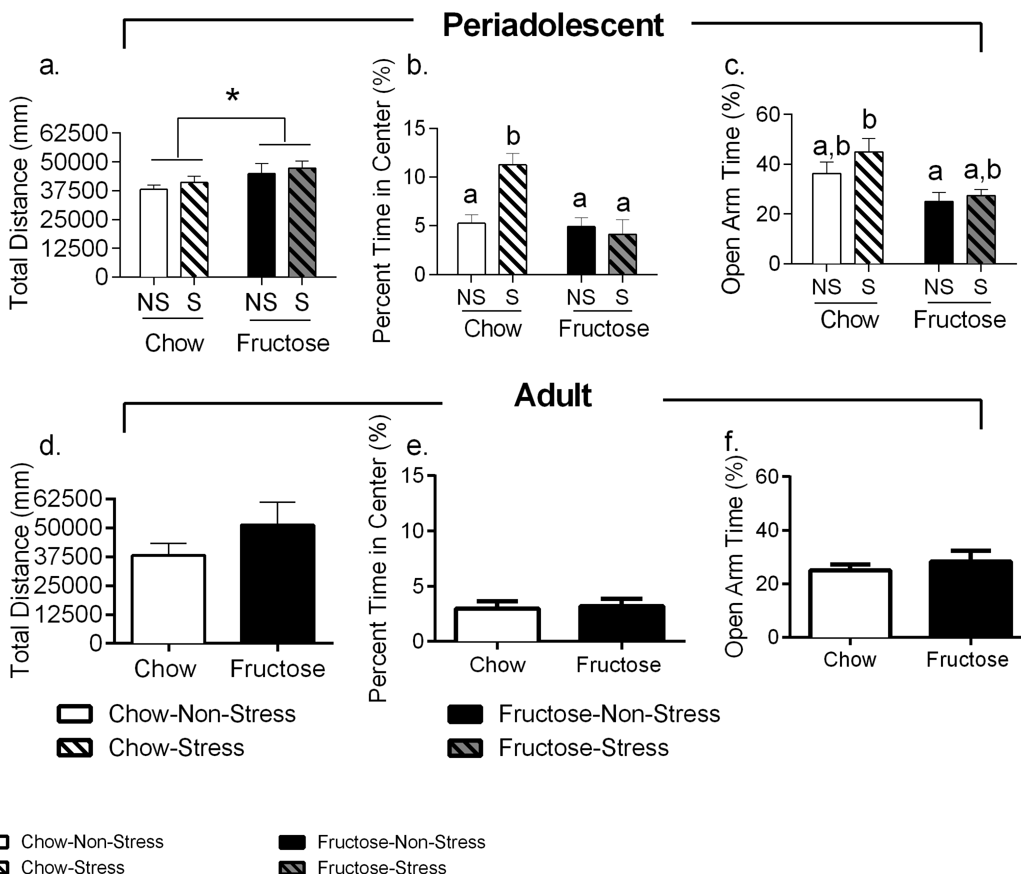
Adolescent Stress and Periadolescent Fructose Affect Weight Gain and Blood Glucose. a. The percent of weight gained during the mid-adolescent stressor (PND37-49) was reduced in the stress cohorts ($p<0.05$) and fructose-fed animals gained more weight during this period ($p<0.05$). b. The fructose diet also significantly increased fasting glucose during mid-adolescence ($p<0.05$**)** irrespective of stress history. c. Glucose administration in the glucose tolerance test raised blood glucose in both chow and fructose stress & non-stress groups ($p<0.05$) as expected. However, fructose diet exacerbated this effect and fructose-fed rats had significantly higher blood glucose in the glucose challenge than the standard chow fed rats ($p<0.05$). Data shown are mean ± SEM; different letters indicate significant differences ($p < 0.05$) as assessed by post-hoc testing. NS=Non-stressed; S=Stressed; C=Saline-injected control; G=Glucose-injected

Figure 5.3.



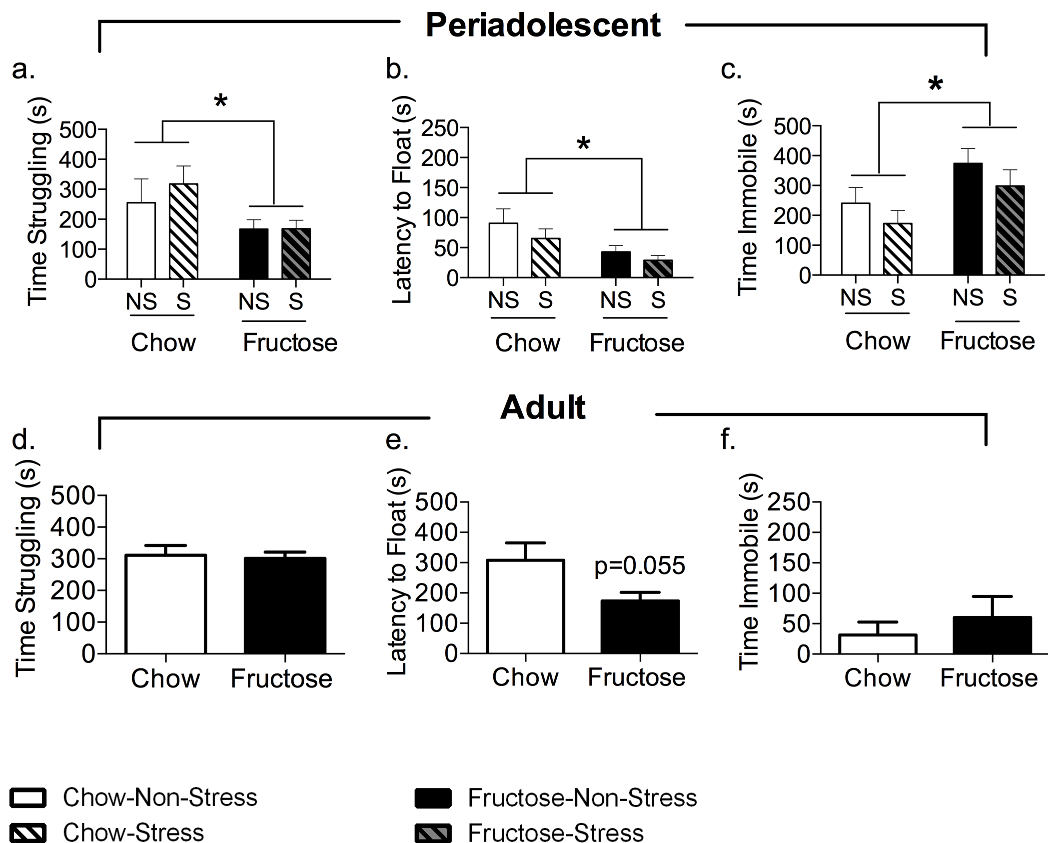
Periadolescent High-Fructose Diet Alters Gene Expression of Hippocampal Glucose Transporter-1 after a Glucose Challenge or Acute Stress The expression of the hippocampal glucose transporter-1 (Slc2a1) was first examined among the non-stressed chow- and fructose-fed animals to assess the effects of the glucose challenge and forced swim test at baseline (**a,b**). **a.** *Slc2a1* expression was not altered by glucose administration. **b.** Forced swim interacted with diet to affect *Slc2a1* expression. Data were next examined among stressed and non-stressed animals within each condition (**c,** saline; **d,** glucose; and **e,** forced swim). The high-fructose diet significantly reduced hippocampal *Slc2a1* expression after both the glucose challenge **(d)** and after the forced swim test **(e)** without an effect of stress. Data shown are mean ± SEM; asterisk indicates an effect of diet and letters indicate post hoc effects with $p<0.05$. C=Chow; F=Fructose; NS=Non-stressed; S=Stressed; FST=Forced Swim Test

Figure 5.4.



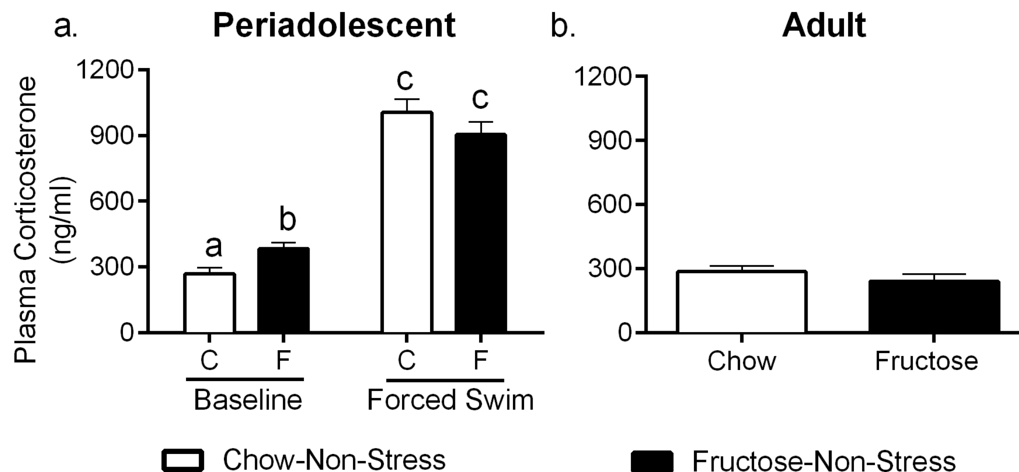
Periadolescent High-Fructose Diet Increases Anxiety-Like Behavior a. Periadolescent fructose-fed rats showed increased activity in the open field. b. Fructose-feeding during periadolescence reduced central tendency, while adolescent stress increased central tendency. c. Periadolescent fructose-fed rats reduced time spent in the open arms of the elevated plus maze. d,e. Rats fed the high-fructose diet in adulthood only did not differ from chow controls in open field or elevated plus maze behavior. Data shown are mean ± SEM; asterisk indicates a main effect of diet while letters indicate significant post-hoc effects with $p < 0.05$. NS=Non-stressed; S=Stressed

Figure 5.5.



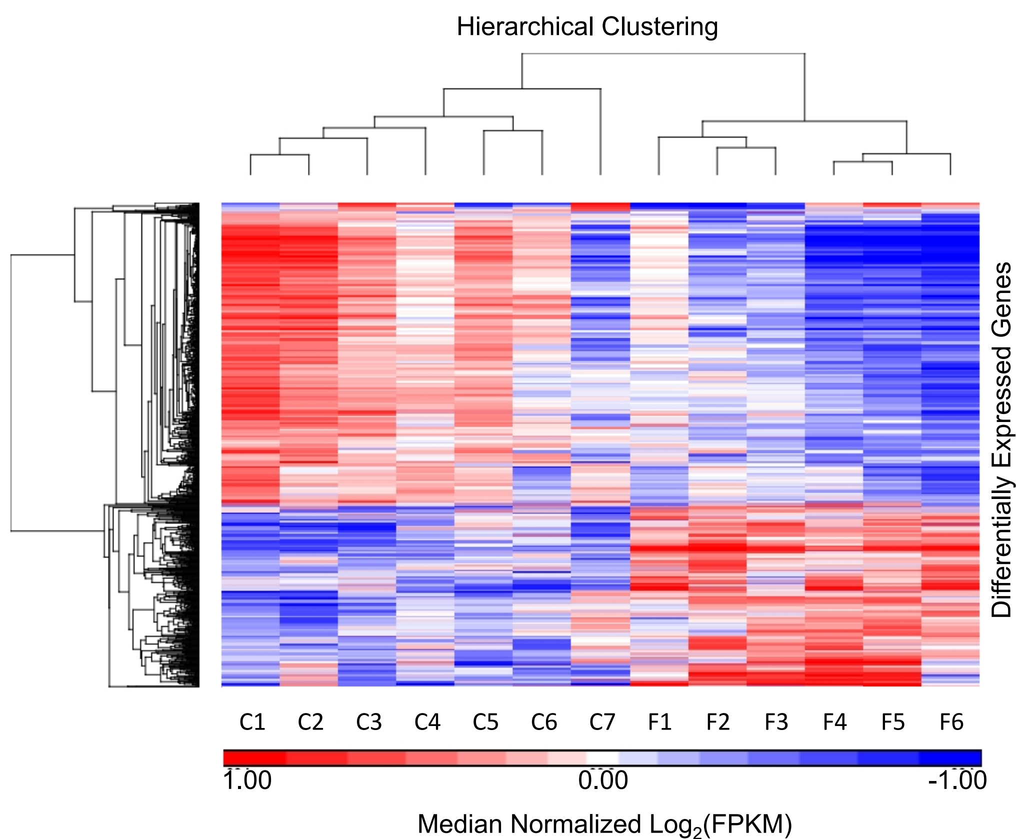
Periadolescent High-Fructose Diet Increases Depressive-Like Behavior a,b,c. Periadolescent fructose-fed rats spent significantly less time struggling, had reduced latency to float, and spent more time immobile in the forced swim test than standard chow fed rats. d,f Rats that consumed the fructose diet in adulthood only did not change in either struggling ($p>0.05$) or immobility ($p>0.05$) in the forced swim. e. However, there was a trend toward reduced latency to float in the adult fructose-fed rats ($t_{14}=2.094$, $p=0.0549$). Data shown are mean ± SEM; asterisk indicates a main effect of diet with $p < 0.05$. NS=Non-stressed; S=Stressed

Figure 5.6



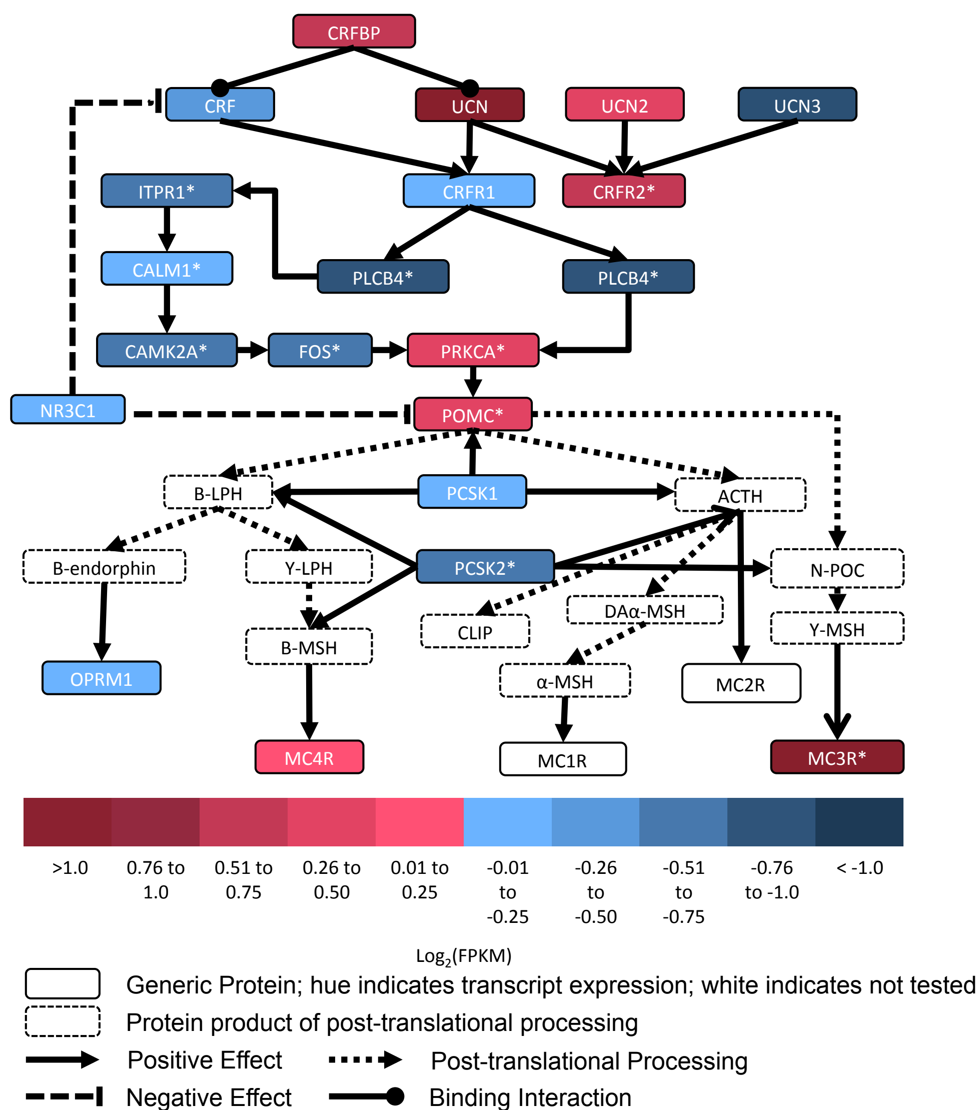
Periadolescent High-Fructose Diet Elevates Baseline Corticosterone a. Plasma corticosterone was analyzed and demonstrated significant increases in basal corticosterone in the periadolescent fructose-fed cohort without differences in the forced swim test. b. Animals fed the high-fructose diet during adulthood only did not differ from chow-fed animals in plasma corticosterone. Data shown are mean ± SEM; different letters indicate significant effects in post-hoc testing with $p < 0.05$. C=Chow, F=Fructose

Figure 5.7



Periadolescent High-Fructose Diet Remodels the Hypothalamic Transcriptome Heatmap of log_2 transformed expression values (FPKM) of the 966 differently expressed transcripts between the two diet conditions (Chow: n=7; Fructose: n=6) in the periadolescent cohort. Zero roughly corresponds to mean expression level.

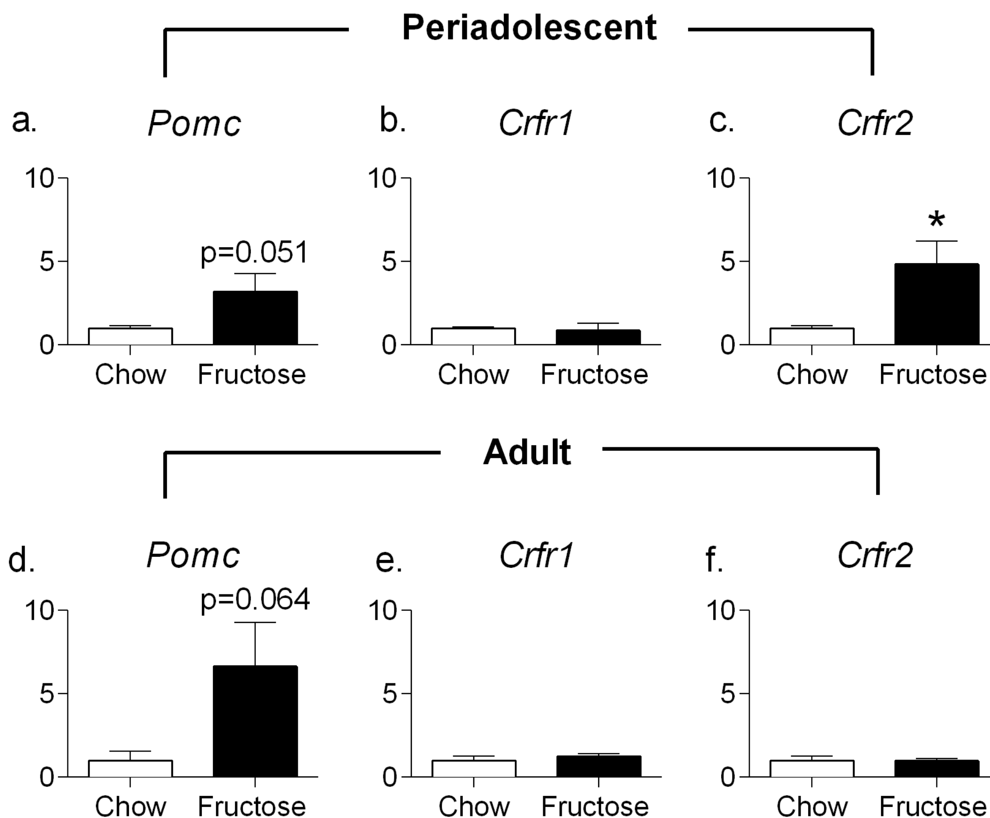
Figure 5.8



Periadolescent High-Fructose Diet Remodels CRF Signaling and POMC Processing

Components of the POMC processing pathway and CRF signaling are combined to show altered genes in these pathways. Hues of the boxes indicate relative transcript expression ($\log_2$(FPKM)) in the fructose-fed animals. An asterisk denotes differentially expressed genes that pass multiple testing corrections.

5.9



High-Fructose Diet Upregulates Hypothalamic Expression of *Pomc* and *Crfr1* when Consumed during Periadolescence but Only *Pomc* when Consumed during Adulthood RT-PCR indicated upregulation of hypothalamic *Crfr2* (c) and a strong trend toward upregulation of *Pomc* (a) in periadolescent fructose-fed animals without an effect on *Crfr1* (b). In animals fed fructose during adulthood only, there was a trend indicating upregulation of *Pomc* (d) but no effect was observed on either *Crfr1* (e) or *Crfr2* (f).

Supplemental Figure 5.1.

Cohort	Post-Natal Day			
Metabolic	23 Wean, Diet			90 ıthanize
Stress 1	23 Wean, Diet	37 Stress	GTT	
Stress 2	23 Wean, Diet	37 Stress	76 OF	92 EPM,FST
Adult				134 EPM,FST

Experimental Timelines. Four cohorts of rats were used in this study. This figure delineates the timelines used for each cohort. The first three cohorts were fed either or a high fructose diet from post-natal day (PND) 25 through adulthood, which begins at PND60 in the rat. Two of these cohorts underwent chronic mixed modality adolescent stress from PND37-48. All subsequent experimental procedures on these cohorts were performed in adulthood. The fourth cohort was fed the high-fructose diet for an equivalent time period but in adulthood only, beginning at PND64. All experimental procedures took place during adulthood in this cohort. All animals were euthanized on the last day noted for their timeline. The diets, ages, experimental uses, and numbers of rats in each cohort are outlined in **Table 5.1**. Abbreviations: GTT: Glucose tolerance test; OF: Open field; EPM: Elevated Plus Maze; FST: Forced Swim Test

Supplemental Table 5.1.

Cohort	Age	Diet	Stress	Experimental Use	N
Metabolic & Vascular Cohort	Periadolescent	Chow	Non-Stress	Caloric Efficiency, Fasting Blood Glucose, Fat Mass, Corticosterone ELISA, PCR (Chapters 5,6,8), Western Blotting (Chapters 6,7)	8
		Fructose			10
		Chow		Caloric Efficiency, Fasting Blood Glucose, Immunohistochemistry (Chapter 8)	8
		Fructose			10
Stress Cohort 1		Chow	Non-Stress	Intra-stress Weight and Fasting Blood Glucose, Saline Control for Glucose Tolerance Test (GTT), Insulin ELISA, RNA-seq, PCR (Chapter 6), Prolactin ELISA (Chapter 7)	7
				Intra-stress Weight and Fasting Blood Glucose, Glucose for GTT, Insulin ELISA	7
			Stress	Intra-stress Weight and Fasting Blood Glucose, Saline Control for GTT, Insulin ELISA	7
				Intra-stress Weight and Fasting Blood Glucose, Glucose for GTT, Insulin ELISA	8
		Fructose	Non-Stress	Intra-stress Weight and Fasting Blood Glucose, Saline Control for GTT, Insulin ELISA, RNA-seq (n=6), PCR (n=6, Chapter 6), Prolactin ELISA (n=6, Chapter 7)	7
				Intra-stress Weight and Fasting Blood Glucose, Glucose for GTT, Insulin ELISA	8
			Stress	Intra-stress Weight and Fasting Blood Glucose, Saline Control for GTT, Insulin ELISA	8
				Intra-stress Weight and Fasting Blood Glucose, Glucose for GTT, Insulin ELISA	8
Stress Cohort 2		Chow	Non-Stress	Intra-stress Weight and Fasting Blood Glucose, Open field, Elevated Plus Maze (EPM), Forced Swim Test (FST), Plasma Corticosterone	14
			Stress		8
		Fructose	Non-Stress		8
			Stress		8
Adult Cohort	Adult	Chow	Non-Stress	Caloric Efficiency, Fasting Blood Glucose, Insulin ELISA, Plasma Corticosterone, Open Field, EPM, FST, PCR (Chapters 5,6), Prolactin ELISA (Chapter 7), Western Blotting (Chapter 7)	8
		Fructose			8

Cohort Distribution and Use. This table displays the use and number of all animals used in this and subsequent studies. All experiments are first discussed in Chapter 5 unless otherwise noted.

Supplemental Table 5.2

	Chow Diet	**High-Fructose Diet**
Company	Lab Diet	Research Diets
Catalog Number	5001	D05111802
Carbohydrate (% kCal)	57	70
Fructose (% kCal)	*0.3*	*55*
Fat (% kCal)	13	10
Protein (% kCal)	30	20
kCal/gram	3.35	3.85

Dietary Composition. The macronutrient distribution of chow and high-fructose diets.

Supplemental Table 5.3.

Week	Periadolescent				Adult			
	Chow		Fructose		Chow		Fructose	
	Mean	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*
0	78	7.8	79	7.3	279	15.5	280	10.5
1	129	11.8	123	7.8	311	24.9	308	15.5
2	190	16.6	181	9.9	342	32.4	342	22.4
3	229	18.7	226	12.3	362	36.3	367	24.0
4	276	22.6	285	17.5	380	40.9	389	27.5
5	323	26.0	332	21.5	405	45.7	411	31.1
6	350	31.0	368	26.2	415	47.6	429	30.0
7	386	32.4	406	31.1	432	53.3	445	31.6
8	414	35.3	443	36.1	445	56.1	457	37.4
9	443	38.6	470	42.4				
10					452	58.0	470	33.6

Mean Weekly Weights in Metabolic Periadolescent and Adult Cohorts. The metabolic periadolescent cohort was euthanized at week nine, while the adult cohort remained on the diet through week ten to match the periadolescent cohort used for behavior. Mean weekly weight and kCal for week 10 used data from weeks 8 and 9, as food weights were not collected on behavioral testing days

Supplemental Table 5.4.

Week	Periadolescent				Adult			
	Chow		Fructose		Chow		Fructose	
	Mean	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*
1	59	4.9	50	4.0	99	7.6	95	7.4
2	80	5.9	72	12.1	105	7.4	96	8.3
3	91	6.2	93	12.3	109	10.0	104	4.7
4	102	8.7	136	28.3	106	6.8	95	9.7
5	117	14.2	108	6.2	112	7.9	101	3.5
6	119	11.2	113	5.7				
7	125	15.1	124	11.2	111	7.1	102	5.2
8	120	8.0	112	7.8	114	8.3	105	5.9
9	108	4.4	100	7.2				
10					96	8.7	98	10.7

Mean Weekly kCal Consumed in Periadolescent and Adult Cohorts. The metabolic periadolescent cohort was euthanized at week nine, while the adult cohort remained on the diet through week ten to match the periadolescent cohort used for behavior. Mean weekly weight and kCal for week 10 used data from weeks eight and nine, as food weights were not collected on behavioral testing days. Food consumption and kCal data for week six was also combined into week seven data.

Supplemental Table 5.5.

Week	Periadolescent Chow		Periadolescent Fructose		Adult Chow		Adult Fructose	
	Mean	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*	*Mean*	*S.D.*
0	100	9.6	90	11.9	106	9.4	108	8.1
1					80	7.2	87	17.5
2					84	13.2	95	17.3
3	72	6.2	81	11.7	79	10.2	82	8.8
4	69	8.5	84	11.8	67	10.1	80	19.0
5	73	7.5	82	7.0	68	8.6	71	14.1
6	63	6.1	70	7.9	79	4.8	93	19.6
7	71	4.9	77	6.3	87	13.4	80	7.2
8	65	6.3	72	7.2	78	18.4	80	14.2
9	71	5.4	79	7.9	78	9.8	81	11.3

Mean Weekly Fasting Blood Glucose in Periadolescent and Adult Cohorts. Fasting blood glucose was not measured for the metabolic periadolescent cohort during weeks one through three to minimize fasting periods that could hamper growth curves during this peak growth period.

Supplemental Table 5.5

Pathway	p Value	q Value	Ratio
Protein folding and maturation_POMC processing	$3.96*10^{-15}$	$2.83*10^{-12}$	17/30
Neurophysiological process_ACM regulation of nerve impulse	$3.06*10^{-10}$	$1.09*10^{-7}$	16/46
Nicotine signaling in dopaminergic neurons, Pt. 1 - cell body	$6.06*10^{-9}$	$1.44*10^{-6}$	15/48
Protein folding and maturation_Posttranslational processing of neuroendocrine peptides	$1.14*10^{-8}$	$2.03*10^{-6}$	15/50
Neurophysiological process_Constitutive and regulated NMDA receptor trafficking	$5.12*10^{-8}$	$7.32*10^{-6}$	16/63
Muscle contraction_GPCRs in the regulation of smooth muscle tone	$1.05*10^{-7}$	$1.25*10^{-5}$	18/83
Signal transduction_Calcium signaling	$1.80*10^{-7}$	$1.83*10^{-5}$	13/45
Development_Angiotensin signaling via STATs	$2.22*10^{-7}$	$1.99*10^{-5}$	11/32
Neurophysiological process_Activity-dependent synaptic AMPA receptor removal	$2.69*10^{-7}$	$2.13*10^{-5}$	15/62
Neurophysiological process_NMDA-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons	$3.25*10^{-7}$	$2.32*10^{-5}$	17/80
Neurophysiological process_GABA-B receptor signaling in presynaptic nerve terminals	$4.50*10^{-7}$	$2.92*10^{-5}$	11/34
Neurophysiological process_Synaptic vesicle fusion and recycling in nerve terminals	$1.13*10^{-6}$	$6.74*10^{-5}$	13/52
Airway smooth muscle contraction in asthma	$2.80*10^{-6}$	$1.54*10^{-4}$	13/56
Ca(2+)-dependent NF-AT signaling in cardiac hypertrophy	$3.47*10^{-6}$	$1.77*10^{-4}$	13/57
Regulation of lipid metabolism_Regulation of lipid metabolism by niacin and isoprenaline	$9.66*10^{-6}$	$4.06*10^{-4}$	11/45
Development_Activation of ERK by Alpha-1 adrenergic receptors	$9.66*10^{-6}$	$4.06*10^{-4}$	11/45
G-protein signaling_Regulation of cAMP levels by ACM	$9.66*10^{-6}$	$4.06*10^{-4}$	11/45
Reproduction_GnRH signaling	$1.07*10^{-5}$	$4.26*10^{-4}$	14/72
Neurophysiological process_Dopamine D2 receptor signaling in CNS	$1.51*10^{-5}$	$5.69*10^{-4}$	11/47
Protein folding_Membrane trafficking and signal transduction of G-alpha (i) heterotrimeric G-protein	$2.27*10^{-5}$	$8.11*10^{-4}$	7/19
Neurophysiological process_Netrin-1 in regulation of axon guidance	$2.52*10^{-5}$	$8.56*10^{-4}$	11/41
Neurophysiological process_Corticoliberin signaling via CRFR1	$2.83*10^{-5}$	$8.86*10^{-4}$	11/50
Neurophysiological process_Constitutive and activity-dependent synaptic AMPA receptor delivery	$2.86*10^{-5}$	$8.86*10^{-4}$	12/59
Immune response_Neurotensin-induced activation of IL-8 in colonocytes	$3.16*10^{-5}$	$9.02*10^{-4}$	10/42
Transport_ACM3 in salivary glands	$3.16*10^{-5}$	$9.02*10^{-4}$	10/42

Pathways Significantly Enriched by Differentially Expressed Genes between Periadolescent Chow & Fructose-fed Cohorts Pathway analysis revealed significant enrichment of 97 pathways with differentially expressed genes; the top 25 pathways are listed. Notably, multiple pathways relating to HPA axis function, including “POMC Processing,” “Post-translational processing of neuroendocrine peptides,” and “Corticoliberin signaling via CRFR1” were

significantly enriched with differentially expressed genes. Ratio refers to ratio of the number of significantly differentially expressed genes vs the total number of genes in the pathway.

Supplemental Table 5.6

Network	p Value	q Value	Ratio
Cell adhesion_Synaptic contact	$6.725*10^{-15}$	$8.878*10^{-13}$	50/184
Signal transduction_Neuropeptide signaling pathways	$1.138*10^{-14}$	$8.878*10^{-13}$	45/155
Development_Neurogenesis_Synaptogenesis	$4.006*10^{-12}$	$2.083*10^{-10}$	45/180
Neurophysiological process_Transmission of nerve impulse	$9.714*10^{-12}$	$3.578*10^{-10}$	49/212
Transport_Calcium transport	$1.147*10^{-11}$	$3.578*10^{-10}$	46/192
Signal transduction_WNT signaling	$6.462*10^{-8}$	$1.680*10^{-6}$	37/177
Muscle contraction	$1.025*10^{-6}$	$2.285*10^{-5}$	34/173
Transport_Potassium transport	$2.064*10^{-6}$	$4.025*10^{-5}$	36/194
Development_Neurogenesis_Axonal guidance	$7.701*10^{-6}$	$1.335*10^{-4}$	39/230
Reproduction_Gonadotropin regulation	$9.985*10^{-6}$	$1.558*10^{-4}$	35/199
Reproduction_GnRH signaling pathway	$6.536*10^{-5}$	$9.269*10^{-4}$	29/166
Neurophysiological process_Long-term potentiation	$2.716*10^{-4}$	$3.531*10^{-3}$	17/82
Neurophysiological process_Circadian rhythm	$3.399*10^{-4}$	$4.079*10^{-3}$	16/76
Neurophysiological process_Corticoliberin signaling	$4.383*10^{-4}$	$4.883*10^{-3}$	12/49
Development_Neuromuscular junction	$7.651*10^{-4}$	$7.957*10^{-3}$	24/147
Development_Neurogenesis in general	$8.430*10^{-4}$	$8.219*10^{-3}$	29/192
Cell adhesion_Cell-matrix interactions	$9.087*10^{-4}$	$8.339*10^{-3}$	31/211
Development_Blood vessel morphogenesis	$1.645*10^{-3}$	$1.412*10^{-2}$	32/228
Neurophysiological process_GABAergic neurotransmission	$1.719*10^{-3}$	$1.412*10^{-2}$	22/138
Transport_Synaptic vesicle exocytosis	$4.043*10^{-3}$	$3.004*10^{-2}$	25/175
Cell adhesion_Attractive and repulsive receptors	$4.043*10^{-3}$	$3.004*10^{-2}$	25/175
Reproduction_Progesterone signaling	$4.479*10^{-3}$	$3.176*10^{-2}$	29/214
Cardiac development_Wnt_beta-catenin, Notch, VEGF, IP3 and integrin signaling	$4.942*10^{-3}$	$3.352*10^{-2}$	22/150
Muscle contraction_Nitric oxide signaling in the cardiovascular system	$5.398*10^{-3}$	$3.508*10^{-2}$	19/124
Proteolysis_Connective tissue degradation	$7.628*10^{-3}$	$4.760*10^{-2}$	18/119

Networks Significantly Enriched by Differentially Expressed Genes between Periadolescent Chow & Fructose-fed Cohorts Network analysis, which can link multiple pathways through interacting genes, demonstrated significant enrichment of 25 networks with differentially expressed genes with the top ranking networks pertaining to synaptic contact and neuropeptide signaling. Ratio refers to ratio of the number of significantly differentially expressed genes vs the total number of genes in the pathway.

Chapter Six

High-Fructose Diet Consumption during Adolescent Development Induces Hypothalamic and Hippocampal Expression of Complement Components

6. Abstract

The deleterious effects of chronic stress on the immune system are well established. Though traditionally thought of in a psychosocial or physical context, chronic stress can also be considered in relationship to nutrition and nutritional status. While a growing body of literature has considered the impact of a high-fat diet on the immune system, a distinct lack of research exists regarding the impact of high-fructose diets. Fructose consumption, which is associated with glucose intolerance, dyslipidemia and hypertension, has increased by over 25% since the 1970s, and adolescents consume the greatest quantity of fructose. In this study, we hypothesized that high-fructose feeding in male rats throughout the juvenile period and into early adulthood would result in multiple inflammatory gene expression changes in the hypothalamus. Whole-transcriptome sequencing (RNAseq) revealed that of the over 2000 genes that were differentially expressed between the two diet groups, inflammatory and immune pathways ranked most highly for enrichment. In particular, both alternative and classical complement pathways were significantly enriched by differentially expressed genes. These data demonstrate that consumption of a diet high in fructose during the peri-adolescent period substantially remodels the hypothalamic transcriptome, specifically with respect to complement factor expression. Alterations in complement pathways have implications for both the response to immune challenges and synaptic maturation in the brain.

6.1. Introduction

The negative effects of chronic developmental stress on immune function and inflammation are well established (Danese, Pariante, Caspi, Taylor, & Poulton, 2007; Pace et al., 2006). Traditionally, chronic stress is conceptualized as repeated exposure to psychological or physical stressors such as social stress (McCormick et al., 2008; McCormick et al., 2011), abuse (Neigh et al., 2009), or trauma (Heim et al., 2008). However, any perturbation to homeostasis that activates the hypothalamic-pituitary-adrenal (HPA) axis fits the definition of a stressor as originally defined by Hans Selye (Hans Selye, 1950). Thus, changes in energy homeostasis can be considered physiologic stressors. In the context of nutrition, chronic stress has been described in relationship to undernutrition such as during the Dutch Famine (de Rooij et al., 2006; Roseboom, de Rooij, & Painter, 2006) and in other instances of starvation (Sparen et al., 2004). Chronic stress in the context of overnutrition has also been described for obesity (Pecoraro et al., 2004; Tamashiro et al., 2009). In Chapter Five, we established the potential for a high-fructose diet, consumed during adolescent development, to alter HPA output and act as a chronic stressor. Here, we examine the effects of this dietary stressor on inflammatory factors in stress-responsive brain regions.

Obesity and metabolic dysfunction are associated with elevated inflammation. Obesity leads to activation of c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathways (Hotamisligil, 2005; Wellen & Hotamisligil, 2005), resulting in elevated Tumor Necrosis Factor (TNF) and interleukin-6 in rodent liver and adipose tissue (Park et al., 2010) as well as human adipose tissue (Kern, Ranganathan, Li, Wood, & Ranganathan, 2001). Similarly, white adipose tissue shows elevated expression of cytokines macrophage inflammatory protein-1α and monocyte chemotactic protein-1 in both genetically obese mice and in diet-induced obesity mouse models (H. Xu et al., 2003). While the effects of diet on central inflammation are less studied, high fat models increase hypothalamic expression of

inhibitor of NFκB-β (IKKβ) (Posey et al., 2009) and maternal obesity increases hippocampal expression of cluster of differentiation molecule 11b (CD11b) and toll-like receptor-4 (Bilbo & Tsang, 2010). Similarly, a high-fat high-fructose diet has been shown to elevate both hypothalamic and hippocampal expression of interleukin-1β (IL1β) and CD11b (Marissal-Arvy et al., 2013).

However, a significant gap exists in the literature with respect to the chronic effects of fructose feeding on neuroinflammation and broader cerebral gene expression, particularly in the population most affected by fructose consumption, namely, adolescents. Like obesity, fructose feeding itself can promote activity of the JNK and NFκB inflammatory cascades in hepatic tissue (Rutledge & Adeli, 2007) and fructose directly promotes production of reactive oxygen species in peripheral tissue, given its stronger reducing capacity relative to glucose (Kaneto et al., 1996). Fructose consumption also stimulates endothelial cell inflammation, upregulating intercellular adhesion molecule-1 (Glushakova et al., 2008) as well as angiotensin-1 receptor and vascular adhesion molecule 1 (Nyby, Abedi, Smutko, Eslami, & Tuck, 2007). More recent studies have identified potential inflammatory effects of fructose feeding in cerebral tissue, as well; two separate groups have observed increases in hippocampal IL6 mRNA (Djordjevic, Bursac, Velickovic, Vasiljevic, & Matic, 2015) or protein (Hsu et al., 2015) after consumption of a high-fructose diet. Both of these groups also reported impaired hippocampal function, either in a spatial memory task (Hsu et al., 2015) or a reduction in polysialylated neural cell adhesion molecule, a marker of synaptic plasticity, in hippocampal synaptosomes (Djordjevic et al., 2015). The high-fructose model used in this study increases adiposity while reducing basal metabolism (**Chapter Five**), which can result in increased adulthood weight (**see Chapter Eight).** Thus, a high-fructose diet may have the capacity to alter the inflammatory response through both direct effects and indirect mechanisms mediated through metabolic dysfunction.

In the present study, we combined the use of whole transcriptome sequencing (RNAseq) with quantitative real-time PCR to assess the effects of high fructose consumption initiated during periadolescence and maintained in adulthood on inflammatory gene expression in the hypothalamus and hippocampus in the male rat. We next determined whether these effects were also seen after high-fructose diet consumption initiated in adulthood. Specifically, we hypothesized that high-fructose feeding during periadolescence would have a more extensive impact on neuroinflammatory gene expression than consumption throughout adulthood alone, given the periadolescent-specific vulnerability to metabolic and hormonal disruption previously observed. In addition, given the literature implicating diet-induced hippocampal inflammation in altered learning and memory (Hsu et al., 2015) as well as synaptic plasticity (Djordjevic et al., 2015), we examined the relationship between expression of inflammatory genes in the hippocampus and the hypothalamus to pre- and post-synaptic markers.

6.2. Materials and Methods

6.2.1 Animal Husbandry. The cohorts used in these experiments were the same as the periadolescent and non-stress and adult cohorts in Chapter Five. In brief, timed pregnant Wistar rats (n=19) were obtained on gestational day 12 from Charles River (Wilmington, MA) while adult male Wistar rats (n=16, PND 56) were obtained from Charles River (Wilmington, MA). Shipping stress during puberty can alter behavioral outcomes (Laroche et al., 2009a) but shipping of pregnant dams has not been shown to alter developmental outcomes without the introduction of a pharmacologic challenge (Ogawa et al., 2007b); thus, shipping was conducted during in utero development to produce the periadolescent cohort. Animals for the adult only diet exposure were obtained from Charles River as adults and acclimated to colony conditions for seven days prior to introduction of the high fructose diet (Capdevila et al., 2007).

Rats were housed on a 14:10 reverse light: dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). Pups were culled to 8 per litter on postnatal day (PND) 3 and rats were weaned on PND 23 (n=29). All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

6.2.2. Diet. As previously described, either two days post-weaning (Periadolescent Cohort; PND 25; n=29) or at PND 64 (Adult Cohort, n=16), male rats were assigned to either the Lab Rodent Diet 5001 (n=23) or a high-fructose diet (n=24). Rats remained pair-housed throughout the study. The fructose diet used (Research diets D05111802) was 55% fructose while the standard chow used was 0.30% fructose. Both diets were supplemented with comparable levels of vitamins and minerals deemed necessary for rodent health, and were reviewed by veterinary staff and approved by IACUC. Blood glucose was tested weekly after an overnight fast by tail prick using a *Freestyle* glucometer. Animal weights were also taken concurrently with glucose readings. Food consumption was measured daily. All metabolic effects of the diet, as well as effects on plasma corticosterone, have been previously described (Chapter Five).

Periadolescent Cohort 1 was used for the RNA sequencing analysis and a portion of PCR validation (chow n=7; fructose n=6) while periadolescent Cohort 2 was used to replicate and extend validation through PCR as well as immunoblotting (chow=8, fructose=10). The same Adult cohort was used for both PCR and immunoblotting. Details of each cohort are listed in **Table 6.1.**

6.2.3 Whole-Transcriptome RNA-Sequencing. As previously described, RNA was extracted from the hypothalamus of each rat in the periadolescent Cohort 1 in order to assess the effects of a high-fructose diet on the hypothalamic transcriptome. These animals were rapidly decapitated one hour after saline injection (as part of a glucose tolerance test; see Chapter Five), trunk blood was collected, and brains were removed and immediately frozen before dissection on dry ice. The whole hypothalamus was lysed and homogenized using Trizol RNA Extraction

reagent (Life Technologies, Grand Island, NY) and QiaShredder (Qiagen, Valencia, CA). RNA was extracted with an RNEasy kit from Qiagen. RNA purity and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). 1 µg of total RNA was used to build mRNA sequencing libraries and a paired-end 100 bp sequencing reaction performed on an Illumina HiSeq 1000 (Illumina, San Diego, CA) generating ~25 million reads per sample. Raw sequence reads were mapped to the most recent RAT assembly (RGSC5.0) using the STAR aligner (Dobin et al., 2013). Data FPKM normalized and differential expression examined using the Cufflinks and CuffDiff software suite (Trapnell et al., 2013; Trapnell et al., 2010). All data discussed in this document have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE56238 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56238.

6.2.4 Pathway Analysis. Pathway analysis of differentially expressed genes established from RNA sequencing was performed using MetaCore (Thomson Reuters, New York City, NY), a web-based computational platform for analysis of high-throughput molecular data (Ekins, Nikolsky, Bugrim, Kirillov, & Nikolskaya, 2007). The MetaCore database of pathways, networks, diseases, and individual protein-protein, protein-DNA, and protein-RNA interactions is one of the most comprehensive databases, containing over 4.5 million individual findings that are manually annotated based on literature reviews from over 2,700 peer-reviewed journals.

As described in O'Connor et al(O'Connor, Schultze-Florey, Irwin, Arevalo, & Cole, 2014), differential expression was identified as transcripts either exceeding a 1.25 or inferior to a 0.75 fold change in expression in chow vs. fructose-fed rats. No statistical testing was applied at the level of individual genes in RNA-seq as this study had no *a priori* single-gene hypothesis and was not designed to detect statistically significant associations between single gene transcripts and diet. The list of differentially expressed transcripts were used to serve as an intermediary input for higher-order bioinformatics that have their own False Discovery Rate (FDR) adjusted p values (q values) less than 0.05. Thus, this list of differentially expressed genes was uploaded

into MetaCore and mapped onto known pathways. Lists of pathway "folders" or groups as well as pathways that were significantly enriched with a greater than expected ratio of altered genes were obtained, and specific genes in each pathway were probed for further analysis of function and expression.

6.2.5 Quantitative RT-PCR For tissue collection in comparison to the periadolescent animals, rats in the Adult cohort (both Chow and Fructose) were sacrificed by rapid decapitation on PND133, trunk blood was collected, and brains were removed and immediately frozen at -80°C. This time-point was chosen because it provided a comparable time of diet exposure in relationship to the periadolescent group. In addition, periadolescent Cohort 2 was collected to extend validation of the RNA-sequencing. These animals were euthanized with Euthasol® and perfused with saline for two minutes, trunk blood was collected, and brains were frozen before dissection on dry ice. Saline perfusion of periadolescent Cohort 2 allowed for differentiation between inflammatory factors in the blood versus cerebral tissue.

To determine whether the effects of the high-fructose diet extended to a different developmental period and from the hypothalamus to the hippocampus, the hippocampi were dissected from the brains of rats fed during periadolescence and adulthood on both diets. The whole hypothalamus from Cohort 1 and the Adult cohort, one half of the hippocampus from Cohort 2, and the left hippocampus from all rats were lysed and homogenized using Trizol RNA Extraction reagent (Life Technologies, Grand Island, NY) and QiaShredder (Qiagen, Valencia, MA). RNA was extracted with an RNEasy kit from Qiagen. RNA concentration and purity was then assessed with a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). RNA was standardized and then reverse-transcribed using the High Capacity RNA to cDNA kit (Life Technologies, Grand Island, NY). cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized to 10 pg/µl. Rat TaqMan Gene Expression Assays were purchased from Life Technologies (Grand Island, NY) with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively: *C4b*

(Rn01525746_g1), *Cfb* (Rn01526084_g1), and *C1qa* (Rn01519903_m1). These components were chosen based on their expression in RNAseq analysis in the hypothalamus in the periadolescent cohort and as representative of the classical (*C1qa, C4b*), lectin (*C4b*) and alternative (*Cfb*) complement pathways.

The following two-step RT-PCR cycling conditions were used on the 7900HT Sequence Detection System (Applied Biosystems): $50^{o}C$ (2 min), $95^{o}C$ (10 min), 40 cycles of $95^{o}C$ (15 s) and $60^{o}C$ (1 min). The housekeeping genes *Ubc* (Rn01789812_g1), *Actb* (Rn00667869_m1), and *Hprt1* (Rn01527840_m1) were run as endogenous controls. After assessment of suitability as housekeeping genes in our samples, *Ubc, Actb,* and *Hprt1* were standardized using the geometric mean of the cycle threshold values (calculated as described in (Vandesompele et al., 2002)) for analyses involving the hypothalamus while only *Hprt1* was used as a housekeeping gene in the hippocampus due to significant ($p>0.05$) variability in the other housekeeping genes across groups when assessed by ANOVA.

Relative gene expression of individual samples run in triplicate (with coefficient of variation cut-off set to 4%) was determined by the comparative $\Delta\Delta C_T$ quantification method with fold change to standard chow of a given developmental cohort. All TaqMan gene expression assays are guaranteed to have 90 - 100% amplification efficiency as determined by the genome-aided probe and primer design pipeline and reported in the “Amplification Efficiency of TaqMan Gene Expression Assays” Application Note 127AP05-03 from Life Technologies.

6.2.6 Immunoblotting. For analysis for synaptic markers in fructose and chow-fed rats, the right hippocampus from Cohort 2 were lysed and homogenized in homogenization buffer (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $MgCl_2$, 0.5% Triton-X 100, pH 7.4) and both protease and phosphatase inhibitors (1:100 dilution, Sigma-Aldrich, St. Louis, MO) by mechanical disruption with a TissueLyserII (Qiagen, Venlo, Netherlands). Protein concentration was assessed using a Pierce BCA Assay Kit (Life Technologies, Carlsbad, CA) and standardized to 3 ug/ul. Protein extracts (30 ug) were reduced by heating in 2x Laemmli sample buffer

(BioRad, Hercules, CA) with β mercaptoethanol, then loaded onto a 4-15% Bis-Tris gel (BioRad, Hercules, CA) and separated by gel electrophoresis. The gels were blotted onto polyvinylidene difluoride membranes and blocked in 7.5% milk in tris-buffered saline with Tween-20 (TBS-T) for one hour. Blots were incubated overnight in primary antibody in 3% bovine serum albumin in TBS-T, washed, then incubated in the appropriate HRP-linked secondary antibody in 2.5% milk in TBS-T, and visualized with SuperSignal WestFemto Maximum Sensitivity Substrate (Life Technologies, Carlsbad, CA). Between primary antibodies, blots were stripped for 30 minutes at 37^0C with a mild stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH 2.2). The primary antibodies used were synaptophysin (1:500, Ab8049, Abcam, Cambridge, MA) and PSD95 (1:2000, 7E3-1B8, Thermo Fisher Scientific, Rockford Illinois); the secondary antibodies used were HRP labeled goat anti-mouse IgG (1:2000, sc2005, Santa Cruz Biotechnology, Dallas, Texas) and HRP labeled goat anti-rabbit IgG (1:5000, sc2004, Santa Cruz Biotechnology, Dallas, Texas), while β-actin (1:50,000, A3854, Sigma Aldrich, St. Louis, MO) was used as a loading control. Optical density of bands was quantified using ImageJ (National Institutes of Health, Bethesda, MD) and the density of each synaptic marker was normalized to the density of β-actin for that sample.

6.2.7 Statistical Analysis. Graphpad Prism (Version 6.0) and R Studio (Version 0.98.1049) were used for statistical analysis of RNA-seq output, PCR and immunoblotting data. For comparisons of these measures between Chow and Fructose groups, unpaired two-tailed Student's t-tests, Analysis of Variance (ANOVA) tests, and linear regression modeling were performed with α=0.05. Holm-Sidak post-hoc testing was performed when appropriate after ANOVA testing.

6.3. Results

6.3.1 High-fructose diet consumption remodels hypothalamic gene expression. Genome-wide transcriptional profiling of hypothalamic RNA from rats fed either standard chow or a high-fructose diet throughout the periadolescent period identified 17,366 transcripts mapping to distinct named genes. Of these, 2,639 transcripts or 15.2% showed a ≥ 1.25 or ≤ 0.75 fold change mean expression level. Among these differentially expressed transcripts, 908 transcripts were downregulated and 1,731 transcripts were upregulated **(Figure 6.1)**.

6.3.2 Hypothalamic immune response and inflammatory pathways are significantly enriched with transcripts altered by fructose consumption. Pathway map folder enrichment by differentially expressed transcripts was determined using GeneGo MetaCore software suite (Thompson Reuters) with q<0.05. Thirty map folders were significantly enriched by differentially expressed transcripts (**Table 6.2**). Of these, over 14% of the transcripts in the "Inflammatory Response" and 18% of those in the "Immune Response" related processes were significantly affected by the high-fructose diet. .

6.3.3 Hypothalamic expression of classical, lectin-induced, and alternative complement pathways are significantly enriched with transcripts altered by fructose consumption. Pathway enrichment by differentially expressed transcripts was determined using GeneGo MetaCore software suite (Thompson Reuters) with q<0.05. Forty-three pathways were significantly enriched by differentially expressed transcripts (**Table 6.3**). While multiple immune and inflammatory related pathways were significantly enriched, it was notable that the "Classical Complement Pathway," the "Lectin Induced Complement Pathway", and the "Alternative Complement Pathway," were significantly enriched (q<0.05), with at least 25% of the genes in each pathway affected. Relative expression levels of genes in these pathways are depicted in **Figure 6.2**.

6.3.4 Rats fed a high-fructose diet during adolescent development have increased expression of complement components C4b and Cfb. To validate expression of two the complement components identified as upregulated in the hypothalamus of the fructose-fed

periadolescent animals by RNA sequencing, qRT-PCR was performed in this cohort (Cohort 1) using primers for *C4b* (involved in the classical and lectin-binding pathways) and *Cfb* (involved in the alternative pathway). Both of these genes had significantly increased expression in the fructose-fed Cohort 1 relative to their chow-fed peers (**Figure 6.3a,b**; *C4b*: t_{11}=14.62; p<0.0001 *Cfb*: t_{11}=7.793; p<0.0001).

To further replicate these findings and to determine the extent to which inflammatory factors in the blood might affect expression of complement components, a second periadolescent cohort (Cohort 2) was fed either fructose or chow during the same time period as Cohort 1. This cohort was saline-perfused before brains were collected, and half of the hypothalamus and one hemisphere of the hippocampus were dissected for qRT-PCR. Complement components *C4b* and *Cfb* were examined as before, and expression of *C1qa* (specific to the classical pathway) was also assessed. In this cohort, *C4b* was significantly elevated in the hypothalamus of fructose-fed animals (**Figure 6.5b**, t_{14}=2.896; p=0.0117), but neither C1qa (**Figure 6.5a**, t_{13}=0.1806; p=0.8594) nor Cfb (**Figure 6.5c**, t_{14}=0.8525; p=0.4083) were significantly changed.

6.3.5 Rats fed a high-fructose diet only in adulthood do not show increased hypothalamic expression of complement components C4b and Cfb. To determine whether the effects of the high-fructose on expression of complement pathways were specific to diet consumption during the periadolescent time period, gene expression was analyzed by qRT-PCR in a cohort of rats that was fed the same high-fructose diet for a comparable length of time but beginning in adulthood (described in Materials in Methods). Hypothalamic gene expression of complement components *C4b* and *Cfb* was assessed in this adult cohort. No significant differences in expression were observed for either gene (**Figure 6.3c,d;** *C4b*: t_{14}=0.9236; p=0.3713; *Cfb*: t_{14}=1.680; p=0.1151).

6.3.6 Increased expression of complement components C4b and Cfb extends from the hypothalamus to the hippocampus in rats fed a high-fructose diet during periadolescence but not adulthood To assess the regional specificity of the high-fructose diet effects on genes in the

complement pathways, expression of *C4b* and *Cfb* was evaluated by qRT-PCR in the left hippocampi of both periadolescent Cohort 1 animals as well as the Adult Cohort. Expression of both components was significantly upregulated in the hippocampus of the rats that were fed throughout the periadolescent period (**Figure 6.4a,b**; *C4b*: t_{11}=4.442; p=0.0010; *Cfb:* t_{11}=2.352; p=0.0384). However, neither component was significantly altered by diet in the hippocampus of the rats that were only exposed to the high-fructose diet in adulthood (**Figure 6.4c,d**; *C4b*: t_{14}=0.5002; p=0.6247; *Cfb:* t_{14}=0.0411; p=0.9678).

In the replication cohort used to determine the extent to which inflammatory factors in the blood might affect expression of complement components (Cohort 2), hippocampal *Cfb* was significantly increased in fructose-fed animals (**Figure 6.5f,** t_{14}=2.373; p=0.0325), and there was a compelling trend for an increase in *C1qa* (**Figure 6.5d**, t_{15}=1.997; p=0.0643). Expression *C4b* (**Figure 6.5e**, t_{14}=0.2812; p=0.7826) was not altered in the hippocampus in this cohort.

6.3.7 Synaptophysin and post-synaptic density 95 are not differentially expressed in the hippocampus of periadolescent fructose-fed rats Hippocampal protein extracts from saline-perfused rats fed either chow or fructose during adolescent development (Cohort 2) were separated by gel electrophoresis, transferred to a PVDF membrane, and probed for the presynaptic marker, synaptophysin, as well as the postsynaptic marker, post-synaptic density 95 (PSD95). Neither synaptophysin (**Figure 6.6a**, t_{15}=0.0169, p=0.9867) nor PSD95 (**Figure 6.6b,** Kolgomorov-Smirnoff D=0.2857; p=0.8038) differed in normalized density between chow- and fructose-fed rats.

6.3.8 Expression of complement factors predicts expression of synaptic markers in the hippocampus Linear regression modeling was used to determine whether hippocampal expression of complement factors, diet group, and/or plasma corticosterone would predict hippocampal expression of either synaptophysin or PSD95 among the rats in Cohort 2. Plasma corticosterone values were calculated as described and previously reported in Chapter 4. For each model, non-normalized values (i.e., CT values and non-normalized optical density values)

were used to prevent confounding. Models were initially inputted and assessed for minimal Akaike Information Criterion (AIC) using a stepwise algorithm. The stepwise AIC method is a model selection method to determine which predictor variables should be retained for a minimum adequate model to describe the dependent variable (Yamashita, Yamashita, & Kamimura, 2007). Based on this algorithm, the minimum adequate model to predict hippocampal synaptophysin density included the final variables corticosterone, *C1qa* CT, and *Cfb* CT (**Table 6.4**). In this model, only *Cfb* CT significantly predicted synaptophysin (B=-2.7060, t_8=-2.303, p=0.0502), indicating that lower *Cfb* expression (higher CT value) indicate higher synaptophysin density. There was also a trend for *C1qa* CT to predict synaptophysin (B=1.1143, t_8=3.047, p=0.0159), indicating a potential effect for lower *C1qa* expression (higher CT) to indicate lower synaptophysin density. The minimum adequate model to predict hippocampal PSD95 included the final variables corticosterone, *C4b* CT, and *Cfb* CT (**Table 6.5**). In this model, corticosterone (B=-0.0163, t_7=3.556, p=0.0093), *C4b* CT (B=-3.2940, t_7=-3.777, p=0.0069), and *Cfb* CT (B=-1.9938, t_7=-2.783, p=0.0272) all significantly predicted PSD95 density. Given the negative sign of each coefficient, higher corticosterone, lower *C4b* expression, and lower *Cfb* expression predict lower PSD95 density.

6.4. Discussion

Our findings that a periadolescent high-fructose diet remodels the hypothalamic transcriptome with a substantial impact on immune response and inflammatory pathways in general, and complement pathways in particular, adds to the growing evidence that dietary manipulations can have dramatic effects on cerebral gene expression (Carlin, George, & Reyes, 2013) and neuroinflammation (Bilbo & Tsang, 2010). Our data indicate that the periadolescent period confers a unique susceptibility to the neuroinflammatory effects of a high-fructose diet in both the hypothalamus and hippocampus. Collectively, these data demonstrate a novel

neuroinflammatory effect in the field of neuro-nutrition and highlight the interactions between development and diet to alter adult cerebral gene expression.

In the present study, RNA-seq analysis revealed that all three complement pathways (classical, lectin, and alternative; **Table 6.3; Figure 6.2**) were significantly enriched with differentially expressed gene transcripts in the hypothalamus of the fructose-fed periadolescent cohort. The complement system consists of approximately 20 proteins, normally present in serum, that play an essential role in our innate immune response. The three primary roles of complement are cell lysis, generation of pro-inflammatory mediators, and opsonization (Levinson, 2012). The three arms of the complement system differ in target recognition but share in common C3 activation (Bonifati & Kishore, 2007). Once thought to be immune privileged, the central nervous system is now known to contain many features of the immune system, including complement proteins (Bonifati & Kishore, 2007; Sardi et al., 2011). Complement proteins are expressed in both neurons and glia, and glia are a major source of complement during both development and neurodegenerative disease (Stephan, Barres, & Stevens, 2012). Components C1 inhibitor, C4, and Factors B and H are also produced in the endothelial cells of brain microvessels (Piroli et al., 2007), which may be another important source of central complement that can play a role in neurological response to stress and injury.

Fructose consumption has been shown to induce a peripheral pro-inflammatory response, including NFκB activation and increased TNF expression in liver (B. S. McEwen, Gould, & Sakai, 1992) and kidney (Pervanidou & Chrousos, 2011). More recent studies have also indicated a pro-inflammatory response of fructose feeding, with potential increases in hippocampal IL6 mRNA (Djordjevic et al., 2015) or protein (Hsu 2015) after consumption of a high-fructose diet. The data presented here extend these findings by demonstrating that fructose consumption can have a widespread effect on the hypothalamic transcriptome with a particular impact on inflammatory pathways (**Tables 6.2 & 6.3**). This is consistent with the concept of fructose as a pro-inflammatory stressor and moreover with complement as a critical mediator of

the innate response, as the classical, lectin, and alternative complement pathways were upregulated in the hypothalamus of the fructose-fed periadolescent cohort. While not the only immune-related pathways affected – leukocyte chemotaxis and interferon antiviral response were also significantly enriched, among others – the complement pathways demonstrated a pattern of upregulation that could be significant for understanding the effects of dietary manipulation on neuroinflammation and the innate immune response in particular.

To assess whether the effects of fructose on complement component expression were specific to consumption during the periadolescent time period, hypothalamic gene expression for components *C4b* (C4, basic form) and *Cfb* (Factor B) was analyzed by qRT-PCR in both the same periadolescent cohort used for RNA-sequencing as well as an adult cohort that was exposed to either chow or a high fructose diet for the same time-period as the periadolescents, but during adulthood only. These specific genes were selected based on their expression levels in the RNA-sequencing experiment and also due to their representative roles in different complement pathways. *C4b* is common to both the lectin-induced and classical pathways while *Cfb* plays a role in the alternative pathway. Once cleaved, a subunit of both C4 and Factor B helps form the C3 convertase for their respective pathways (Levinson, 2012). While both hypothalamic *C4b* and *Cfb* were increased by fructose consumption in the periadolescent cohort, neither were affected by fructose consumption in adulthood only (**Figure 6.3**). Thus, the data presented indicate that early components of each pathway – that would further activate and increase expression of later components - are not altered in the hypothalamus by fructose consumption throughout adulthood. This evidence is consistent with the conception of adolescence as a “critical period” of development (E. I. Knudsen, 2004; Simpson & Davies, 1994; Sisk & Zehr, 2005).

The hypothalamus was selected as a region of interest as it plays an essential role in food intake and energy homeostasis (Marty et al., 2007). As the primary locus of response for alterations in metabolic homeostasis, the dramatic remodeling of the hypothalamic transcriptome by periadolescent fructose consumption is not entirely unexpected. Therefore, we sought to

establish whether the effects of fructose consumption extended beyond the hypothalamus. The hippocampus was selected as a secondary region of interest as it is a key modulator of the stress response (Vannucci, Koehler-Stec, et al., 1998), especially in adolescence (Piroli et al., 2007; Simpson & Davies, 1994) and also a region susceptible to diet-induced inflammatory changes (Bilbo & Tsang, 2010; Marissal-Arvy et al., 2013). Quantitative RT-PCR revealed that expression of both *C4b* and *Cfb* was increased in the hippocampus of periadolescent but not adult fructose-fed rats (**Figure 6.4**). These data demonstrate that the effects of the high-fructose diet extend beyond the hypothalamus and are additionally consistent with the concept of adolescence as a "critical period" of development.

As further validation, expression of complement genes was examined in a second cohort of rats (Cohort 2) that underwent the same dietary manipulation as the first periadolescent cohort (Cohort 1). However, rats in Cohort 2 were saline-perfused prior to brain removal to evaluate whether the same changes in expression would be present in the absence of cerebral blood, given the high expression of complement components in plasma (Morgan & Gasque, 1997). In this cohort, only *C4b* was significantly elevated in the hypothalamus of fructose-fed rats, while *Cfb* was significantly increased in the hippocampus (**Figure 6.5**). In addition, *C1qa*, the first subcomponent of the C1 complex that initiates the classical cascade, showed a trend for a 1.5-fold increase in hippocampal expression in fructose-fed rats (**Figure 6.5**). While these data do not fully replicate the findings from the non-saline-perfused cohort, they do indicate that increases in cerebral complement expression still occur in periadolescent fructose-fed rats even without the contribution from plasma complement.

Alterations in the complement cascade have been previously characterized in the context of obesity and metabolic dysfunction. Adipose tissue produces key regulatory proteins of the alternative complement pathway, including Factor D (also known as adipsin) and Factor B, as well as C3, the common target for cleavage of all three pathways (Choy, Rosen, & Spiegelman, 1992). Omental tissue in obese men also has high expression of C2, C3, C4, C5 and Factor 7

(Gabrielsson et al., 2003). Obese young children show increases in plasma C3 (Cianflone, Lu, Smith, Yu, & Wang, 2005), and circulating C3 is linearly correlated with waist circumference in adolescents of both sexes. Type II diabetic patients also demonstrate increased circulating complement proteins Factor B and C3 (Fujita et al., 2013). Given this literature, several recent clinical studies have examined the potential role of serum and plasma complement expression as a predictor of metabolic dysfunction and have found that increased circulating C3 is a risk factor for coronary heart disease (Onat et al., 2005) and the metabolic syndrome (Onat, Hergenc, Can, Kaya, & Yuksel, 2010). It is possible that fructose-induced increase in central complement expression reflects peripheral changes, especially given that greater changes in hypothalamic and hippocampal complement occurred in fructose-fed animals that had not been saline-perfused **(Figures 6.3, 6.4, and 6.5**). While we do not have data regarding the state of peripheral complement in the high-fructose diet animals, we would hypothesize for future studies that fructose-fed rats would show elevated circulating complement protein expression as well as increased complement expression in adipose tissue, particularly given the increased peri-renal and epididymal fat masses in these animals (**Chapter Five**).

Both neuroinflammation and chronic stimulation of the HPA axis, as seen in the periadolescent high fructose animals (current chapter and Chapter 4), are associated with altered synaptic function. While acute stress or glucocorticoid administration promotes glutamate release (Popoli, Yan, McEwen, & Sanacora, 2012) and can enhance hippocampal long-term potentiation (LTP), chronic stress or prolonged or elevated glucocorticoid administration can impair hippocampal LTP (Joels, Pu, Wiegert, Oitzl, & Krugers, 2006). Likewise, neuroinflammatory components can be either adaptive or maladaptive in their effect on synaptic function. Activated microglia release nitric oxide, which blocks reuptake of glutamate into the presynaptic neuron and promotes excessive activation of NMDA receptors on the postsynaptic neuron (Rao, Kellom, Kim, Rapoport, & Reese, 2012). This results in excitotoxicity that can damage synaptic proteins. However, some inflammatory factors, notably complement components, may also play a role in

normal synaptic development (Stevens et al., 2007). Expression of complement may be essential for synaptic pruning, as deficiency in either C1q, the initiating factor of the classical pathway, or C3, which is cleaved by C3 convertases formed from either C4 or Factor B, results in defects in CNS synapse elimination in the visual system (Stevens et al., 2007). Developing synapses, which express C1q and C3, are tagged for elimination by activated C3b (Stephan et al., 2012; Stevens et al., 2007). Microglia, which express the C3 receptor, are recruited to the tagged synapse for engulfment and removal (Stephan et al., 2012). This effect may become maladaptive in the context of injury or disease, however, and promote synaptic stripping (Kettenmann, Kirchhoff, & Verkhratsky, 2013). In contrast, an additional, immune-system-independent mechanism has also been proposed to play a role in the mechanism through which C3 proteins promote synaptic stripping. One 2013 study demonstrated reduced synaptic stripping in C3-/- mice after sciatic nerve lesion but an equivalent glial activation to mice deficient in major histocompatibility class Ia that displayed increased synaptic stripping after the same injury (Berg, Zelano, Thams, & Cullheim, 2013). C3aR expression on neural progenitor cells may promote neurogenesis, and reduced neurogenesis has been observed in C3-/- mice after cerebral ischemia independent of microglial cell recruitment or astrocyte activation (Rahpeymai et al., 2006). In sum, complement appears to play multiple roles in synaptic plasticity through both immune and non-immune mediated mechanisms.

In the present study, there were no differences between diet in density of synaptophysin, an integral membrane protein involved in synaptic vesicle formation on presynaptic neurons (Wiedenmann & Franke, 1985), and PSD95, a scaffolding protein of the membrane-associated guanylate kinase (MAGUK) family at the postsynaptic density. However, we did not isolate subcellular components and could not differentiate whether there were differences in whether synaptic proteins, most notably synaptophysin, were located in synaptosomes or located in endosomes targeted for degradation. Such subcellular fractionation may have enabled a more nuanced differentiation based on diet type, or at least better enrichment of protein.

However, corticosterone and hippocampal complement gene expression did predict synaptophysin and PSD95 density even in these poorly enriched samples. Corticosterone was negatively associated with density of both synaptophysin and PSD95, though this relationship was only significant for PSD95 (**Table 6.5**). This is consistent with the literature demonstrating that chronically elevated corticosterone can alter synaptic function (Joels et al., 2006) and reduce expression of synaptic markers (Cohen et al., 2011). Intriguingly, while high hippocampal *Cfb* expression significantly predicted low synaptophysin levels, though high *C1qa* expression trended toward high synaptophysin density (**Table 6.4**). In addition, high *Cfb* and high *C4b* expression were both significantly associated with high PSD95 expression (**Table 6.5**). While this may at first seem counterintuitive, it is in fact consistent with the literature demonstrating high colocalization of complement factors with synaptic markers including PSD95 and the synaptic vesicle protein SV2 (Stevens et al., 2007), including an increase of C1q associating with increased PSD95 (Stephan et al., 2012), and may be reflective of an active process. Recent work from Stephan et al (Stephan et al., 2012) has shown that while both C1q and C3 contribute to synaptic pruning, deficiency in these components induces opposing effects on synaptic potentiation. This research has received greater attention due to the growing awareness of the role of increased complement in both neurologic disease, such as Alzheimer's disease (Eikelenboom, Hack, Rozemuller, & Stam, 1989; McGeer, Akiyama, Itagaki, & McGeer, 1989) and multiple sclerosis (Ingram et al., 2012), as well as injury, including traumatic brain injury (Stahel, 2004), spinal cord injury (Galvan et al., 2008), and stroke (Engstrom, Hedblad, Janzon, & Lindgarde, 2007; E. D. Pedersen, Waje-Andreassen, Vedeler, Aamodt, & Mollnes, 2004; Szeplaki et al., 2009). While more research will be necessary to elucidate the role of Factor B and C4 in synaptic activity and their relationship to diet and/or altered HPA axis output, it is possible that the elevated complement in periadolescent fructose-fed animals represents one potential mechanism linking diet to neurologic disease risk. Future studies should explore the possibility that that an

increase in neurological disease associated with metabolic dysfunction may have a common cause rooted in neuroinflammation, particularly with respect to the complement cascade.

One caveat to the presented findings is that while complement expression predic

In sum, our findings demonstrate that periadolescent high-fructose feeding substantially remodels the hypothalamic transcriptome and increases complement component expression in the hypothalamus and hippocampus. Periadolescent fructose consumption affected multiple immune and inflammatory pathways in the hypothalamus, with notable upregulation in classical, lectin-induced and alternative complement pathways. Though the effects on complement extend to the hippocampus for the periadolescent cohort, these effects do not extend to rats that consumed the high fructose diet only during adulthood, indicating developmental specificity of the effects of fructose consumption. Upregulation in complement in the periadolescent cohort may have implications for the later response to stroke (see Chapter Eight;(Cavusoglu et al., 2007; Engstrom et al., 2007)), synaptic maturation in the brain (Stevens et al., 2007), and potential neurodegeneration (Bonifati & Kishore, 2007; Eikelenboom et al., 1989). These data add to the growing evidence that diet-induced metabolic dysfunction during a critical period can promote neuroinflammation. Given the widespread influence of the high-fructose diet on expression of cerebral inflammatory factors, this study highlights the profound influence of diet on neuroimmunology and paves the way for future research in this field.

Table 6.1. Cohort Distribution and Use.

Cohort	Age	Diet	Stress	Experimental Use	N
Cohort 1	Periadolescent	Chow	Non-Stress	RNA-seq, PCR (*C1qa, C4b, Cfb*) Rapidly Decapitated after Fasting and Saline Injection for Glucose Tolerance Test	7
		Fructose			6
Cohort 2	Periadolescent	Chow	Non-Stress	PCR (*C1qa, C4b, Cfb*), Western Blotting (Synaptophysin, PSD95) Saline-Perfused, Non-Fasting	8
		Fructose			10
Adult Cohort	Adult	Chow	Non-Stress	PCR (*C1qa, C4b, Cfb*) Rapidly Decapitated, Non-Fasting	8
		Fructose			8

Cohort Distribution and Use. This table lists the number and use of animals in Chapter 6. For a full description of all uses of each cohort, see Chapter Five.

Table 6.2.

Rank	Map Folder	p value	FDR	Ratio
1	Depression	$1.05*10^{-20}$	$6.27*10^{-19}$	237/1267
2	Asthma	7.0110^{-20}	$2.10*10^{-18}$	324/1963
3	Cell differentiation	$2.27*10^{-17}$	$4.55*10^{-16}$	219/1205
4	Immune system response	$2.42*10^{-16}$	$3.63*10^{-15}$	212/1176
5	Stem cells	$9.50*10^{-11}$	$1.14*10^{-09}$	75/328
6	Prostatic Neoplasms	$3.37*10^{-09}$	$3.37*10^{-08}$	170/1044
7	Dermatitis, Allergic Contact	$7.81*10^{-09}$	$6.69*10^{-08}$	179/1126
8	Tissue remodeling and wound repair	$2.13*10^{-08}$	$1.60*10^{-07}$	103/563
9	Neurodegeneration in Multiple sclerosis	$1.65*10^{-07}$	$1.10*10^{-06}$	219/1498
10	Calcium signaling	$2.01*10^{-07}$	$1.21*10^{-06}$	95/529
11	Inflammatory response	$2.55*10^{-07}$	$1.39*10^{-06}$	148/933
12	Ovarian cancer	$5.50*10^{-07}$	$2.75*10^{-06}$	215/1487
13	Neurotransmission	$1.34*10^{-05}$	$6.18*10^{-05}$	130/853
14	Melanoma	$3.11*10^{-05}$	$1.33*10^{-04}$	198/1427
15	Colorectal Neoplasms	$5.78*10^{-05}$	$2.31*10^{-04}$	175/1248
16	Cardiac Hypertrophy	$8.88*10^{-05}$	$3.33*10^{-04}$	45/236
17	Multiple myeloma	$4.00*10^{-04}$	$1.32*10^{-03}$	179/1327
18	Vasoconstriction	$4.16*10^{-04}$	$1.32*10^{-03}$	59/355

Immune and Inflammatory Pathway Map Folders Rank Highly in Analysis of Diet-Dependent Differentially Expressed Genes. Listed are the "map folders" (collections of pathways in MetaCore) that were significantly enriched with differentially expressed genes. Ratio refers to ratio of the number of significantly differentially expressed genes vs the total number of genes in the pathway map folder.

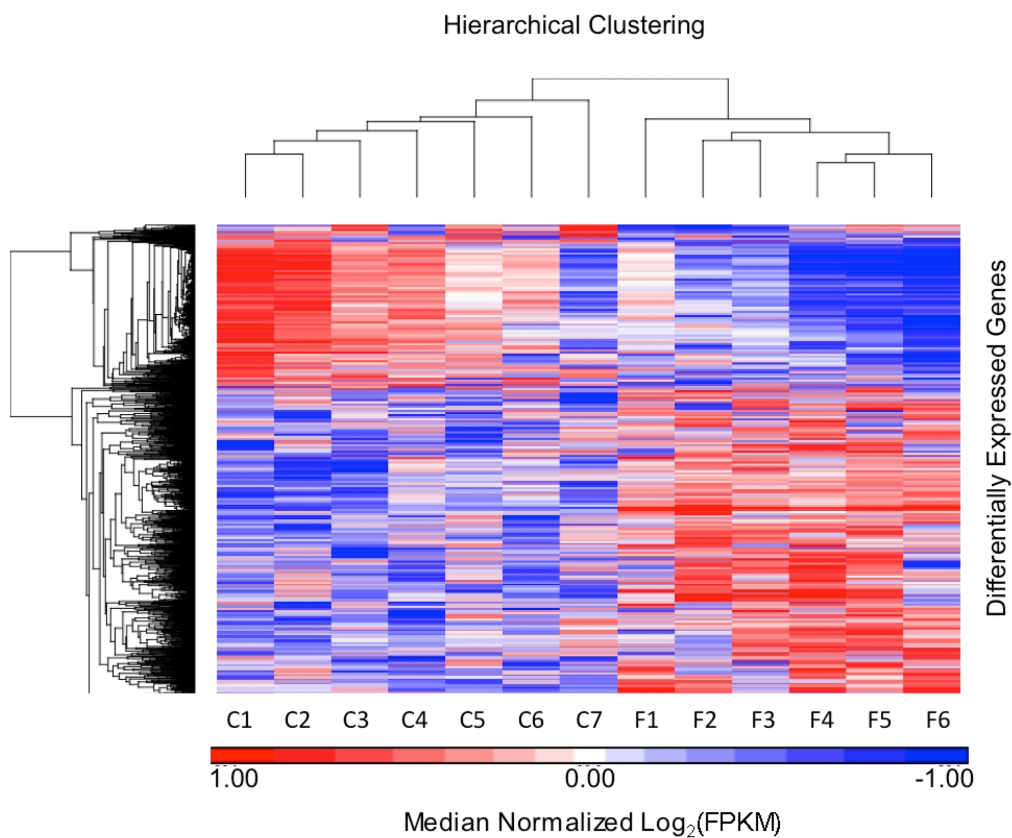
Table 6.3.

Rank	Pathway Map	p value	FDR	Ratio
1	Protein folding and maturation_POMC processing	$2.34*10^{-10}$	$1.80*10^{-07}$	17/30
2	Development_Transcription factors in segregation of hepatocytic lineage	$2.68*10^{-08}$	$1.03*10^{-05}$	15/30
3	Blood coagulation_Blood coagulation	$1.85*10^{-06}$	$3.91*10^{-04}$	15/39
4	Expression targets of Tissue factor signaling in cancer	$2.03*10^{-06}$	$3.91*10^{-04}$	11/22
5	Immune response_Classical complement pathway	$5.26*10^{-06}$	$8.09*10^{-04}$	17/52
6	Neurophysiological process_ACM regulation of nerve impulse	$1.95*10^{-05}$	$2.50*10^{-03}$	15/46
7	Cell adhesion_ECM remodeling	$2.36*10^{-05}$	$2.59*10^{-03}$	16/52
8	Protein folding_Membrane trafficking and signal transduction of G-alpha (i) heterotrimeric G-protein	$3.12*10^{-05}$	$3.00*10^{-03}$	9/19
9	Immune response_Lectin induced complement pathway	$4.52*10^{-05}$	$3.86*10^{-03}$	15/49
10	Immune response_Differentiation and clonal expansion of CD8+ T cells	$5.37*10^{-05}$	$4.13*10^{-03}$	13/39
11	Upregulation of MITF in melanoma	$1.04*10^{-04}$	$7.27*10^{-03}$	12/36
12	Development_Role of proteases in hematopoietic stem cell mobilization	$1.53*10^{-04}$	$9.82*10^{-03}$	8/18
13	Chemotaxis_Leukocyte chemotaxis	$2.72*10^{-04}$	$1.61*10^{-02}$	18/75
14	FGF signaling in pancreatic cancer	$3.50*10^{-04}$	$1.87*10^{-02}$	13/46
15	Development_Beta-adrenergic receptors signaling via cAMP	$3.65*10^{-04}$	$1.87*10^{-02}$	14/52
16	NF-AT signaling in cardiac hypertrophy	$4.33*10^{-04}$	$2.08*10^{-02}$	16/65
17	Transport_FXR-regulated cholesterol and bile acids cellular transport	$5.27*10^{-04}$	$2.14*10^{-02}$	12/42
18	Serotonin modulation of dopamine release in nicotine addiction	$5.27*10^{-04}$	$2.14*10^{-02}$	12/42
19	Nicotine signaling (general schema)	$5.44*10^{-04}$	$2.14*10^{-02}$	8/21
20	Th17 cells in CF	$5.55*10^{-04}$	$2.14*10^{-02}$	14/54
21	Development_Beta adrenergic receptors in brown adipocyte differentiation	$6.16*10^{-04}$	$2.18*10^{-02}$	11/37
22	Development_Growth factors in regulation of oligodendrocyte precursor cell proliferation	$6.22*10^{-04}$	$2.18*10^{-02}$	16/67
23	Development_Melanocyte development and pigmentation	$6.84*10^{-04}$	$2.25*10^{-02}$	13/49
24	Development_Angiotensin signaling via STATs	$7.02*10^{-04}$	$2.25*10^{-02}$	10/32
25	Immune response_Role of HMGB1 in dendritic cell maturation and migration	$7.66*10^{-04}$	$2.36*10^{-02}$	9/27
26	Muscle contraction_Regulation of eNOS activity in cardiomyocytes	$8.24*10^{-04}$	$2.40*10^{-02}$	14/56
27	Nicotine signaling in cholinergic neurons	$8.43*10^{-04}$	$2.40*10^{-02}$	13/50
28	Muscle contraction_GPCRs in the regulation of smooth muscle tone	$9.98*10^{-04}$	$2.67*10^{-02}$	18/83
29	Immune response_Alternative complement pathway	$1.01*10^{-03}$	$2.67*10^{-02}$	11/39
30	Development_Activation of ERK by Alpha-1	$1.04*10^{-03}$	$2.67*10^{-02}$	12/45

	adrenergic receptors			
31	Immune response_Antiviral actions of Interferons	$1.25*10^{-03}$	$3.01*10^{-02}$	13/52
32	Immune response_T cell subsets: cell surface markers	$1.25*10^{-03}$	$3.01*10^{-02}$	13/52
33	Type 2 diabetes (general schema)	$1.35*10^{-03}$	$3.15*10^{-02}$	6/14
34	Neurophysiological process_Dopamine D2 receptor signaling in CNS	$1.57*10^{-03}$	$3.38*10^{-02}$	12/47
35	Development_GH-RH signaling	$1.58*10^{-03}$	$3.38*10^{-02}$	11/41
36	Development_Regulation of lung epithelial progenitor cell differentiation	$1.58*10^{-03}$	$3.38*10^{-02}$	11/41
37	Role of prenatal nicotine exposure in apoptosis and proliferation of pancreatic beta cells	$1.79*10^{-03}$	$3.62*10^{-02}$	9/40
38	Neurophysiological process _Visual perception	$1.79*10^{-03}$	$3.62*10^{-02}$	9/40
39	Nicotine signaling in dopaminergic neurons, Pt. 1 - cell body	$1.91*10^{-03}$	$3.77*10^{-02}$	12/48
40	Resolution of inflammation in healing myocardial infarction	$2.02*10^{-03}$	$3.90*10^{-02}$	8/25
41	Neurophysiological process_Long-term depression in cerebellum	$2.31*10^{-03}$	$4.23*10^{-02}$	12/49
42	Th17 cells in CF (mouse model)	$2.31*10^{-03}$	$4.23*10^{-02}$	12/49
43	Airway smooth muscle contraction in asthma	$2.58*10^{-03}$	$4.61*10^{-02}$	13/56

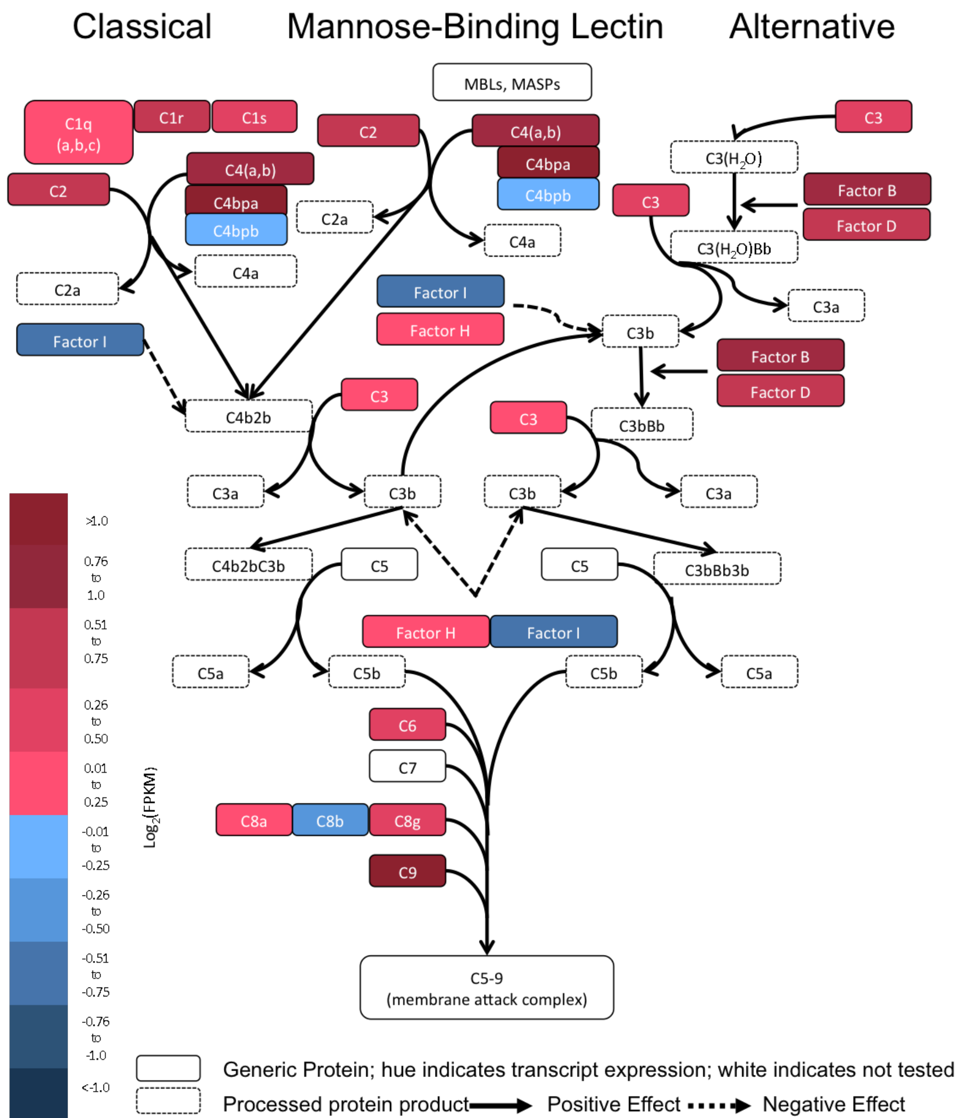
Significantly Enriched Pathways by Diet-Dependent Differentially Expressed Genes Listed are the pathways identified in MetaCore as significantly enriched with differentially expressed genes in the fructose-fed rats. Ratio refers to ratio of the number of significantly differentially expressed genes vs the total number of genes in the pathway.

Figure 6.1.



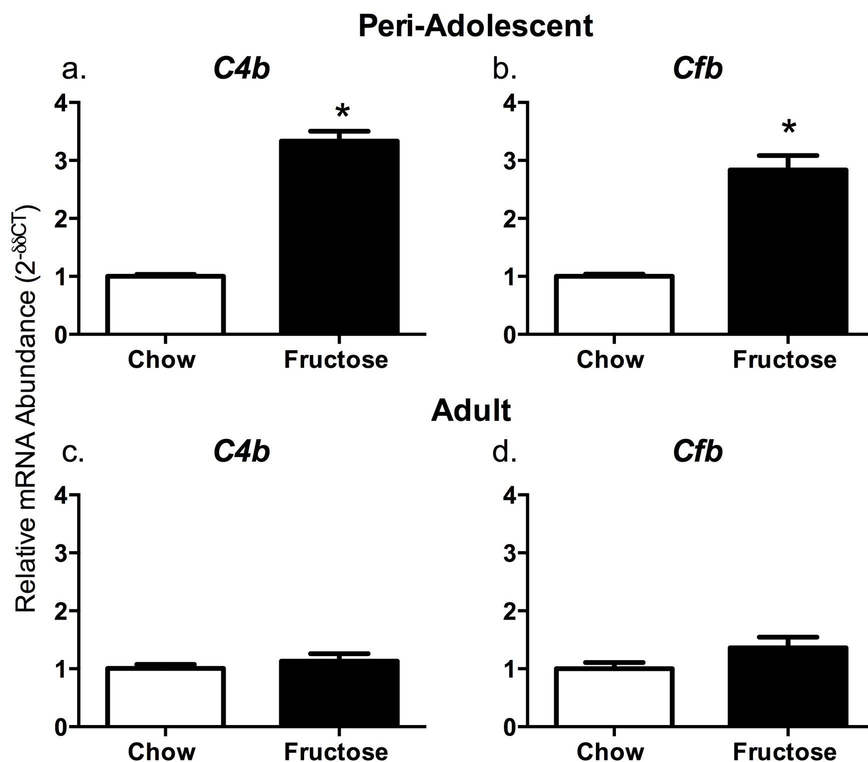
Periadolescent High Fructose Diet Remodels the Hypothalamic Transcriptome Log_2 transformed expression values (FPKM) of the differently expressed genes (at the level of either greater than 1.25 fold or less than 0.75 fold) between the two diet conditions. Zero roughly corresponds to mean expression level.

Figure 6.2.



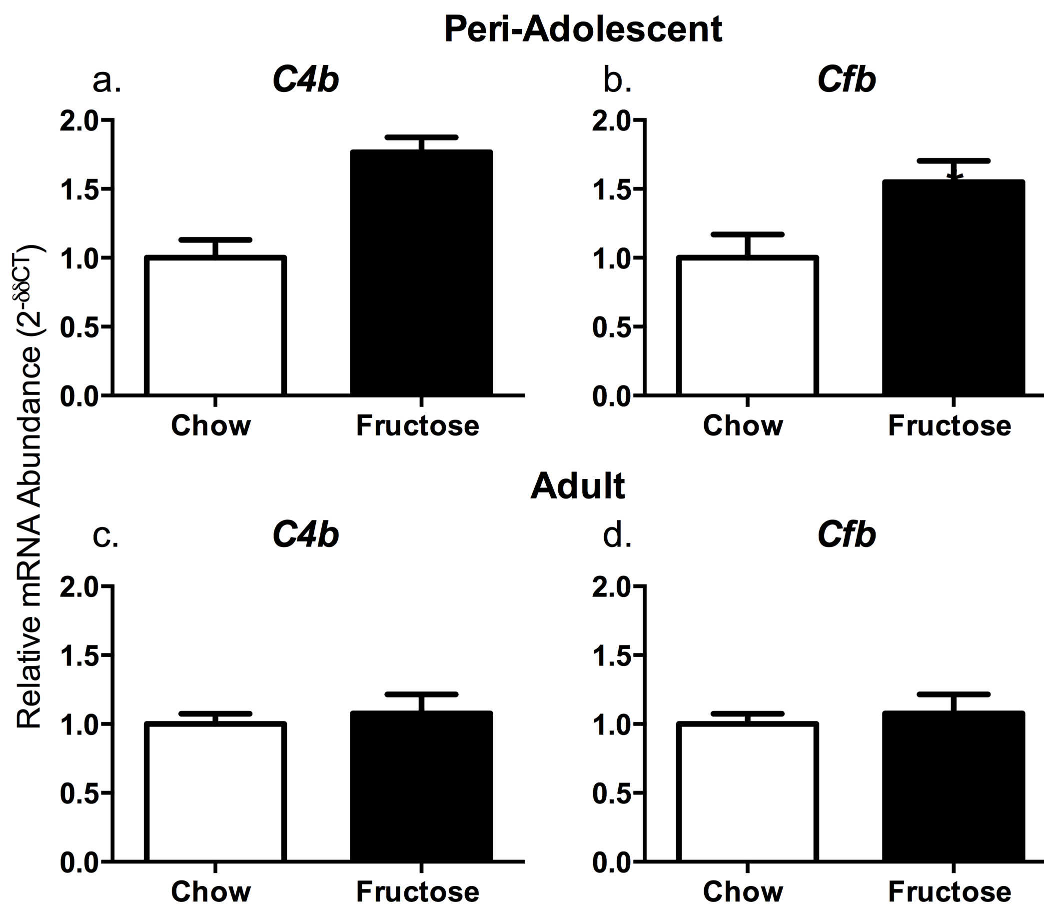
Periadolescent High-Fructose Diet Increases Expression of Gene Transcripts in the in All Three Complement Pathways. All three complement pathways were among the 43 pathways significantly enriched by differentially expressed genes in the standard and fructose-fed periadolescent rats. Here, the pathways have been combined and median $\log_2$ transformed expression values (FPKM) of genes in periadolescent fructose-fed cohort 1 are shown.

Figure 6.3.



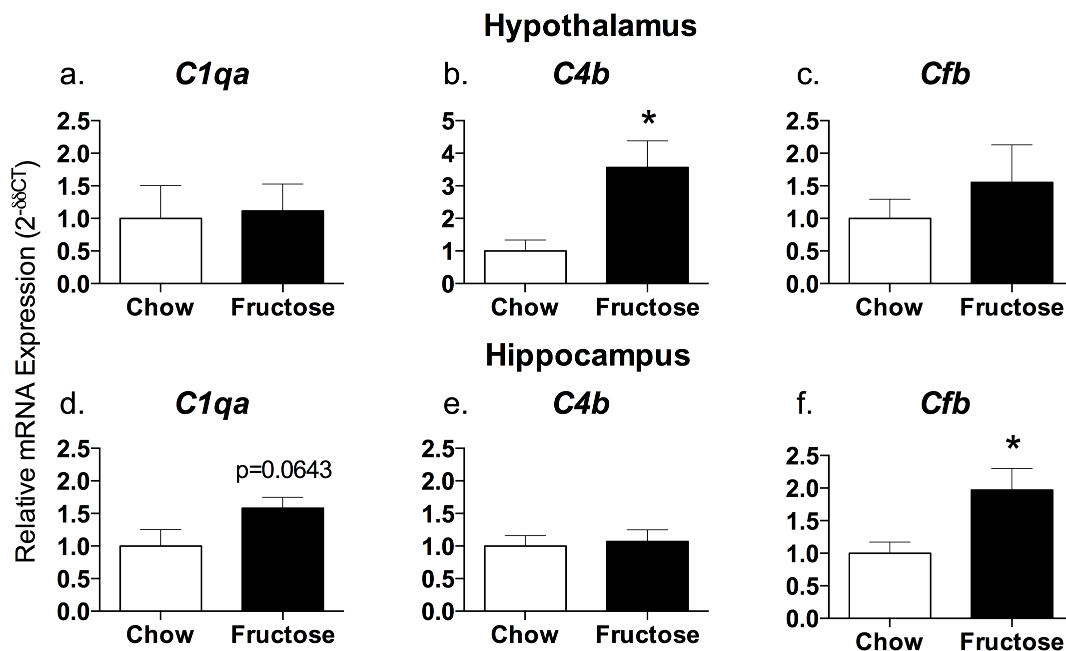
Periadolescent but not Adult Consumption of a High-Fructose Diet Elevates Hypothalamic Expression of Complement Components *C4b* and *Cfb* Expression of both components was significantly upregulated in the hypothalamus of the fructose-fed periadolescent animals (**a,b**). However, neither component was significantly altered by diet in the hypothalamus of the adult cohort (**c,d**). An asterisk indicates a significant effect of diet at $p<0.05$. Data are shown as mean ± SEM.

Figure 6.4.



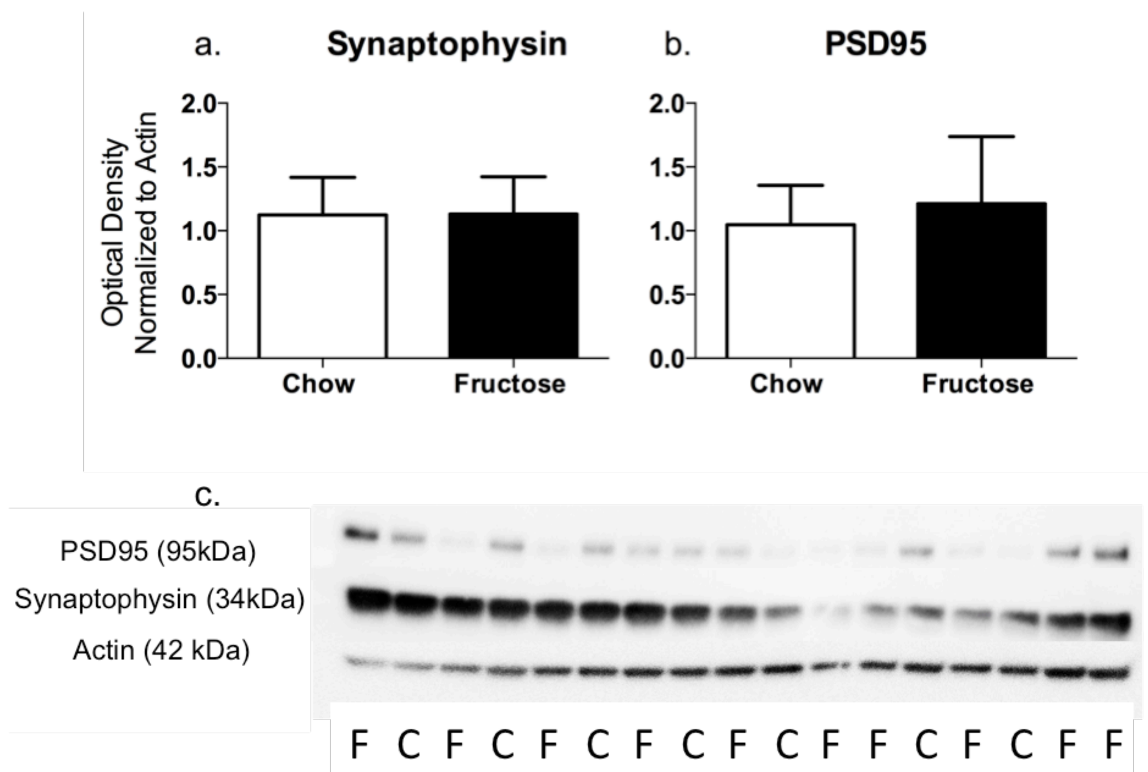
Periadolescent but not Adult Consumption of a High-Fructose Diet Elevates Hippocampal Expression of Complement Components *C4b* and *Cfb* Expression of both components was significantly upregulated in the hippocampus of the fructose-fed periadolescent animals (**a,b**). However, neither component was significantly altered by diet in the hippocampus of the adult cohort (**c,d**). An asterisk indicates a significant effect of diet at $p<0.05$. Data are shown as mean ± SEM.

Figure 6.5.



Hypothalamic C4b and hippocampal Cfb are elevated without the contribution of blood factors In a saline-perfused periadolescent fructose- or chow fed cohort, complement components *C4b* and *Cfb* were examined as before, and expression of *C1qa* (specific to the classical pathway) was also assessed. In this cohort, *C4b* was significantly elevated in the hypothalamus of fructose-fed animals (**b**) but neither C1qa nor Cfb (**a,c**) were significantly changed. In the hippocampus, *Cfb* was significantly increased in fructose-fed animals (**f**), and there was a compelling trend for an increase in *C1qa* (**d**). Expression of *C4b* (**e**) was not altered in the hippocampus in this cohort. An asterisk indicates a significant effect of diet at $p<0.05$. Data are shown as mean ± SEM.

Figure 6.6



Synaptophysin and post-synaptic density 95 are not differentially expressed in the hippocampus of periadolescent fructose-fed rats Neither synaptophysin (**a**) nor PSD95 (**b**) differed in normalized density between chow- and fructose-fed rats. Blots are shown in c. Data are shown as mean ± SEM.

Table 6.4.

Synaptophysin~Variable	**Beta**	**Error**	**t value**	**p value**
(Intercept)	4.9825	0.6507	1.730	0.1220
Corticosterone	-0.6219	0.7232	-0.849	0.4204
C1qa CT	-2.0873	0.9064	-2.303	0.0502
Cfb CT	1.8710	0.6141	3.047	0.0159*

$F_{3,8}=4.165; p=0.0473;$ *Adjusted* $R^2=0.4633;$ *AIC*$=57.0793$

Hippocampal *C1qa* and *Cfb* predict synaptophysin expression. Decreased *Cfb* expression (increased CT value) predicts increased synaptophysin. In contrast, there is a trend that increased *C1qa* expression (decreased CT value) predicts reduced synaptophysin.

Table 6.5.

PSD95~Variable	Beta	Error	t value	p value
(Intercept)	4.4380	0.6254	7.097	0.0019*
Corticosterone	-2.1729	-0.0046	3.556	0.0093*
C4b CT	-2.3735	0.6284	-3.777	0.0069*
Cfb CT	-3.3477	1.2030	-2.783	0.0272*

$F_{3,7}$=16.87; p=0.0014; Adjusted R^2=0.8264; AIC=49.4242

Hippocampal *C4b* and *Cfb* predict PSD95 expression. Decreased corticosterone, increased *C4b* (decreased CT), and increased *Cfb* (decreased CT) all predict an increase in PSD95.

Chapter Seven

High-fructose diet initiated during adolescent development alters hypothalamic expression of mRNA related to dopamine synthesis and signaling without affecting protein expression in male rats

7.0 Abstract

Consumption of simple sugars such as fructose can promote behavioral phenotypes that resemble features of addition. Combining these phenotypes with evidence that sugar consumption can alter in mesolimbic dopamine signaling, growing interest has arisen into the possibility of "sugar addiction." However, while signaling in the mesolimbic pathway may regulate hedonic drives for consumption, signaling in the hypothalamus regulates the homeostatic drives for consumption. While there is substantial crosstalk between these two systems, hypothalamic dopamine remains understudied in the context of nutrition. The studies in this chapter examine the effects of periadolescent high-fructose feeding on expression of genes related to dopaminergic signaling, and explores whether these changes extend to mesolimbic brain regions and/or to animals fed a high-fructose diet through adulthood only. The data in this study indicate that periadolescent fructose feeding altered expression of multiple genes related to dopaminergic signaling within the hypothalamus, nut these effects did not extend to adults or to other brain regions at the level of protein. Future research will be necessary to disentangle the degree to which changes are state dependent (ie, in a fed- vs fasting-state) and to explore other potential signals that may shed light on hypothalamic-mesolimbic communication in the context of feeding.

7.1. Introduction

Given the worldwide obesity epidemic and its adverse effects on health outcomes, a growing interest has arisen on the topic of “food addiction” and “sugar addiction” in both the popular press and the scientific literature (Avena, Rada, & Hoebel, 2008; Lutter & Nestler, 2009). A Google search for “food addiction” produces over 30 million results, while a Google search for “sugar addiction” produces over 7.5 million results. As evidence of the growing scientific interest in the topic, a PubMed search for “food addiction” resulted in 21.7 publications per 100,000 in 2013, a 2.68 fold increase relative to the 8.1 publications per 100,000 associated with these terms in 2003. Similarly, a PubMed search for “sugar addiction” resulted in 7.1 publications per 100,000 in 2013, a 1.99 fold increase relative to the 3.5 publications per 100,000 associated with these terms in 2003 (Corlan, 2004).

Palatable food consumption, and consumption of simple sugars in particular, promotes behavioral phenotypes that resemble features of addiction, including “bingeing,” “withdrawal,” “craving,” and cross-sensitization . In rats, one week of intermittent sucrose access results in increased sucrose consumption (over three times the original quantity consumed) during the first hour of access, as well as increased chow consumption, potentially resembling a “binge” (Colantuoni et al., 2002). With respect to withdrawal, administration of the opioid antagonist naloxone precipitates withdrawal-like symptoms, including teeth chattering and head shaking, in rats given access to intermittent sucrose but not in chow-fed controls, though naloxone or naltrexone administration to human binge-eaters and bulimics does not induce withdrawal-like symptoms (Benton, 2010). Rats will increase lever pressing after glucose deprivation, indicating “craving” for simple sugars (Avena, Long, & Hoebel, 2005), and intermittent sucrose induces behavioral cross-sensitization to amphetamine in male rats (Avena & Hoebel, 2003)though similar effects were not observed in female rats exposed to a high-fructose diet (Bruggeman et al., 2011).

In addition to these behavioral similarities between drug addiction and what one might term “food addition,” there are multiple physiological similarities in the response to drugs of

abuse and food. Food consumption, and palatable food consumption in particular, produces a "reward" signal that is processed in mesolimbic reward circuitry that interacts with nuclei in the hypothalamus, including the ventromedial and lateral hypothalamus (Lutter & Nestler, 2009; Meguid et al., 2000). At a basic level, dopamine signaling is an essential part of feeding; dopamine-deficient mice (due to selective deletion of tyrosine hydroxylase in dopaminergic neurons) are aphagic, and an intraperitoneal injection of L-dopa will reinstate feeding (Zhou & Palmiter, 1995). However, the precise role of hypothalamic dopamine in modulating feeding is poorly understood. The two primary groups of dopaminergic neurons in the hypothalamus are (1) those descending from the A11 and A13 cell groups in the dorsal hypothalamus that regulate sympathetic preganglionic neurons and (2) the ascending tuberoinfundibular pathway arising from the A12 and A14 cell groups along the wall of the third ventricle (C.B. Saper, 2000), which is best known for its role in regulation of prolactin secretion (K. E. Moore, Demarest, & Lookingland, 1987). With respect to food intake, the ventromedial nucleus of the hypothalamus (VMN) increases dopamine release after food intake, and release is associated with the duration of meal consumption (Meguid et al., 2000). Though stimulation of the lateral hypothalamus (LHA) generally stimulates feeding and promotes a concomitant increase in accumbens dopamine (Hernandez & Hoebel, 1988), stimulation of LHA dopamine by injection of α-melanocyte stimulating hormone reduces food intake (Legrand, Lucas, Breton, Dechelotte, & Fetissov, 2015). It has been proposed that desynchronization of VMN and LHA dopamine may promote satiety (Meguid et al., 2000).

Hypothalamic nuclei can communicate with mesolimbic circuitry, specifically both the nucleus accumbens and the VTA, through melanocortigenic and orexigenic nuclei, which may in turn alter dopamine turnover in these regions (Lutter & Nestler, 2009). Furthermore, the feeding related hormones leptin and ghrelin, known to primarily act within the hypothalamus, can also act on accumbens and VTA dopamine neurons. Leptin may act on dopaminergic and GABAerigic neurons within the VTA to mediate satiety (Fulton et al., 2006), while ghrelin appears to act on

dopaminergic neurons in the VTA to reinforce feeding (Abizaid et al., 2006). Thus, the hypothalamic response to feeding, whether directly via dopaminergic signaling or indirectly through other neuroendocrine mediators, may further influence key regions of the mesolimbic reward circuit.

The hypothalamic and mesolimbic dopamine circuitry are not only implicated in addiction, but in depression as well (Nestler & Carlezon, 2006). Anhedonia as well as neurovegetative symptoms including altered appetite, sleep, and energy are key features of depression (DSM-V). Though these pathways are less often studied that the limbic-hypothalamic-pituitary-adrenal axis in the context of depression, they may play an important role in mediating some of the anhedonic features of depression (Nestler et al., 2002). With the hypothalamus as a key relay center communicating between these regions and also given its role as an essential regulator of appetite, circadian rhythm, and energy (Nestler et al., 2002), elucidating its role in the mesolimbic circuit and relationship to depression could result in new targets for therapy.

Given the literature indicating potential alterations in dopamine signaling and circuitry in response to palatable feeding, as well as the behavioral features seen in rats fed a high-fructose diet during adolescence (Chapter Five), we examined the effect of this diet on hypothalamic mRNA related to dopamine signaling. We next assessed whether these changes corresponded to a change in plasma prolactin, as a proxy for dopamine release in the hypothalamus. Finally, we determined whether a periadolescent high-fructose diet would alter protein expression of tyrosine hydroxylase, dopamine transporter, and vesicular monoamone transporter-2 in multiple mesolimbic brain regions.

7.2. Materials and Methods

7.2.1 Animal Husbandry In this series of experiments, tissue from a subset of the same periadolescent animals as well as from the adult animals in Chapter Five was used. In brief, timed pregnant Wistar rats (n=22) were obtained on gestational day 12 to produce the periadolescent cohort (Chow n=15, Fructose n=16) from Charles River (Wilmington, MA) while male Wistar rats (Chow n=8, Fructose n=8, PND 56) were obtained from Charles River (Wilmington, MA) to produce the adult cohort. Full descriptions of breakdown and use of each cohort are in **Table 7.1.** Rats were housed on a 14:10 reverse light: dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). Litters were culled on postnatal day (PND) 3 to eight pups per litter and weaned on PND 23 (n=134). All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

7.2.2 Diet As in Chapter Five, periadolescent male rats were pair-housed at weaning and two days post-weaning were assigned to either a chow diet (n=15) or a high-fructose diet (n=16). Adult males were assigned to either chow (n=8) or the high-fructose diet (n=8) at PND64. The fructose diet used (Research diets D05111802) is 55% fructose while the standard chow (Lab Rodent Diet 5001) normally used is 0.30% fructose. Both diets were supplemented with comparable levels of vitamins and minerals deemed necessary for rodent health, and were reviewed by veterinary staff and approved by IACUC; macronutrient consistency is listed in **Table 5.2.** Metabolic analyses, including food consumption, weight gain, caloric efficiency, fasting blood glucose, and fat pad mass were assessed as described in Chapter Five.

7.2.3 Whole-Transcriptome RNA-Sequencing Consumption of a high-fructose diet during adolescence alters metabolism, affective and locomotor behavior, and basal plasma corticosterone (see Chapter Five). Given these findings, we examined transcriptional changes induced by periadolescent fructose feeding in a brain region that mediates metabolic, hormonal, and behavioral outputs, namely, the hypothalamus. As previously described, RNA was extracted

from the hypothalamus of a subset of the periadolescent cohort (chow: n=7; fructose: n=6) in order to assess the effects of a high-fructose diet on the hypothalamic transcriptome. These animals were rapidly decapitated one hour after saline injection (as part of a glucose tolerance test; see Chapter Five), trunk blood was collected, and brains were removed and immediately frozen before dissection on dry ice. The whole hypothalamus was lysed and homogenized using Trizol RNA Extraction reagent (Life Technologies, Grand Island, NY) and QiaShredder (Qiagen, Valencia, CA). RNA was extracted with an RNEasy kit from Qiagen. RNA purity and quality was assessed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

As described in Chapter Five, 1 μg of total RNA was used to build mRNA sequencing libraries and a paired-end 100 bp sequencing reaction performed on an Illumina HiSeq 1000 (Illumina, San Diego, CA) generating ~25 million reads per sample. Raw sequence reads were mapped to the most recent RAT assembly (RGSC5.0) using the STAR aligner (Dobin et al., 2013). Data FPKM were normalized and differential expression was determined with a false discovery rate (FDR) <0.05 using the Cufflinks and CuffDiff software suite (Trapnell et al., 2013; Trapnell et al., 2012). All data discussed in this document have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE56238 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56238.

7.2.4 Pathway Analysis As in Chapter Five, pathway analysis of differentially expressed genes established from RNA sequencing was performed using MetaCore (Thomson Reuters, New York City, NY), a web-based computational platform for analysis of high-throughput molecular data (Ekins et al., 2007). The MetaCore database of pathways, networks, diseases, and individual protein-protein, protein-DNA, and protein-RNA interactions is one of the most comprehensive databases, containing over 4.5 million individual findings that are manually annotated based on literature reviews from over 2,700 peer-reviewed journals. Differentially expressed genes discovered through CuffDiff analysis were uploaded into MetaCore and mapped onto known pathways. Lists of pathways that were significantly enriched with a greater than expected ratio of

differentially expressed genes at the FDR < 0.05 were obtained, and specific genes in each pathway were probed for further analysis of function and expression.

7.2.5 Prolactin Analysis As a proxy for hypothalamic dopamine activity, this study examined plasma prolactin levels in the same periadolescent animals used in RNA sequencing as well as in adult animals. Prolactin is a polypeptide hormone secreted by lactotrophs of the anterior pituitary and under dopaminergic regulation (Fitzgerald & Dinan, 2008). Dopamine is reported to be the most important prolactin inhibiting factor found in the hypothalamus (Fitzgerald & Dinan, 2008). Prolactin levels were assessed via ELISA (Alpco, Salem, NH, sensitivity 0-80 ng/ml). For the ELISA, trunk blood was collected in EDTA-coated tubes and centrifuged for plasma collection before storing at -80°C. All samples were run in duplicate, and replicates with a CV greater than 10% were not included in analysis.

7.2.6 Immunoblotting For analysis of proteins related to dopamine signaling and turnover in fructose and chow-fed rats, the following brain regions were selected for immunoblotting: dorsal striatum from the same periadolescent animals used in RNA-sequencing; dorsal striatum from adult animals; and ventral tegmental area (VTA), nucleus accumbens (NAcc), and hypothalamus from the second set of periadolescent animals. All tissue was lysed and homogenized in homogenization buffer (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $MgCl_2$, 0.5% Triton-X 100, pH 7.4) and both protease and phosphatase inhibitors (1:100 dilution, Sigma-Aldrich, St. Louis, MO) by mechanical disruption with a TissueLyserII (Qiagen, Venlo, Netherlands). Protein concentration was assessed using a Pierce BCA Assay Kit (Life Technologies, Carlsbad, CA) and standardized to 3 ug/ul. Protein extracts were reduced by heating in 2x Laemmli sample buffer (BioRad, Hercules, CA) with β mercaptoethanol, then standard quantities were loaded onto a 4-15% Bis-Tris gel (BioRad, Hercules, CA) and separated by gel electrophoresis. The gels were blotted onto polyvinylidene difluoride membranes and blocked in 7.5% milk in tris-buffered saline with Tween-20 (TBS-T) for one hour. Blots were incubated overnight in primary antibody in 5% milk in TBS-T, washed, then incubated in the

appropriate HRP-linked secondary antibody in 2.5% milk in TBS-T, and enhanced with chemiluminescence. Between primary antibodies, blots containing dorsal striatum samples were stripped for 15 minutes at room temperature in Pierce Stripping Buffer (Rockford, IL) while remaining blots were stripped for 30 minutes at 37^{0}C with a mild stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween-20, pH 2.2).

Rabbit polyclonal anti-VMAT2 serum raised against a peptide in the C-terminal region of mouse VMAT2 (CTQNNVQPYPVGDDEESESD) by Covance Custom Immunology Services was provided by the laboratory of Gray Miller (Lohr et al., 2014) and used at a dilution of 1:5000. Additional primary antibodies used were rabbit anti-tyrosine hydroxylase (1:5000; Millipore AB152, Billerica, MA) and rat anti-dopamine transporter (1:5000; Millipore MAB369, Billerica, MA). Appropriate HRP-linked secondary antibodies (Jackson ImmunoResearch and Santa Cruz; all 1:5000) were applied and β-actin (A3854, Sigma-Aldrich, St. Louis) was used as a loading control. The luminescence signal from blots containing dorsal striatum samples was captured on an Alpha Innotech (San Leandro, CA) Fluorochem imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to co-blotted dilution standards of pooled protein from control samples as previously described (Caudle, Colebrooke, Emson, & Miller, 2008). The luminescent signal from blots containing samples from other regions was captured on a G:Box Chemi (Syngene, Frederick, MD). Densitometric analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD) and the density of each synaptic marker was normalized to the density of β-actin for that sample.

7.2.7 Statistical Analysis Graphpad Prism (Version 6.0), R (Version 3.3.1), and R Studio (Version 0.98.1049) were used for statistical analysis and graphing of RNA-sequencing, PCR and immunoblotting data. Analytical measures included Principal Components Analysis (PCA), unpaired two-tailed Student's t-tests, Analysis of Variance (ANOVA) tests, and linear regression modeling. In tests of significance, α was set to 0.05. Holm-Sidak post-hoc testing was performed when appropriate after ANOVA testing.

7.3. Results

7.3.1 Fructose and chow diets determine clustering of the hypothalamic transcriptome

Principal components analysis (PCA) was used to provide another means of assessing genes that drive the differences between chow-fed and fructose-fed animals. When evaluating FPKM expression of each of the 17,366 transcripts mapped to known genes for each animal, the PCA identified two distinct populations, perfectly clustered by diet type (**Figure 7.1**). Principal component 1 (PC1) accounted for 24.7% of the variance in gene expression, while PC2 accounted for 13.0% and PC3 accounted for 11.8% of the variance, collectively accounting for 49.5% of the total variance in gene expression. The ten most influential top- and bottom-loading factors in each PC are shown in **Table 7.2**. Notably, the dopamine transporter gene, *Slc6a3*, was a top-loading factor in each PC, while tyrosine hydroxylase (*Th*) was a top-loading factor in PC1. Additionally, several nicotinic acetylcholine receptor subunits were among the top-loading factors in the three PCs, namely, the nicotinic acetylcholine receptor subunit α5 (*Chrna5*), α6 (*Chrna6*), and β3 (*Chrnb3*). Among the notable bottom-loading genes, oxytocin (*Oxt*), arginine-vasopressin (*Avp*), thyrotrophic releasing hormone (*Trh*), and FEZ family zinc finger 1 (*Fezf1*) were all listed in both PC2 and PC3. The prolactin releasing hormone receptor (*Prlhr*) was a bottom-loading gene in PC2.

7.3.2 Periadolescent fructose feeding alters expression of genes in dopamine signaling pathways As noted in Chapter Four, pathway analysis revealed significant enrichment of 97 pathways with differentially expressed genes (all $q<0.05$; see **Table 5.3**). When ranked by the –log(p-value), “Nicotine signaling in dopamine neurons” was the third highest ranked pathway, and “Dopamine D2 receptor signaling” was the 32nd ranked pathway (both FDR $q<0.01$). Further exploration revealed that multiple genes related to dopamine synthesis and signaling were also differentially expressed in the hypothalamus of periadolescent chow- and fructose-fed animals

(**Table 7.3**), including dopamine decarboxylase (*Ddc*), vesicular monoamine transporter-1 (*Slc18a1*), vesicular monoamine transporter-2 (*Slc18a2*), dopamine receptor-1 (*Drd1*), and dopamine receptor-2 (*Drd2*).

7.3.3 Plasma prolactin is not significantly altered in either periadolescent or adult fructose-fed rats Though diminished, plasma prolactin was not significantly decreased in periadolescent fructose-fed rats (**Figure 7.2a,** t_9 =1.770; p=0.1105). Plasma prolactin was also not significantly changed in adult fructose-fed rats, though it was slightly increased relative to chow fed rats (**Figure 7.2b**, Kolgomorov Smirnov D=0.2500, p=0.9801).

7.3.4 Plasma prolactin predicts dopamine transporter expression, tyrosine hydroxylase, and vesicular monoamine transporter-2 gene in RNA-sequencing To determine the relationship between gene expression of DAT (gene name *Slc6a3*), TH (gene name *Th*), and VMAT2 (gene name *Slc18a2*) and plasma prolactin as well the physiological factors plasma corticosterone, weight, and blood glucose, these variables were entered into linear regression models. Akaike's Information Criterion was examined by stepwise regression in both directions, and all variables remained in each model. As expected, prolactin inversely predicted *Slc6a3* expression (**Table 7.4;** β_5=-1.9848; p=0.0185). Weight (β_5=2.0068; p=0.0435) and plasma corticosterone (β_5=2.445; p=0.0051) were positive predictors of *Slc6a3* expression. The adjusted R^2 for this model was 0.7982 ($F_{4,5}$=9.902; p=0.0135). Prolactin also inversely predicted *Th* expression (**Table 7.5;** β_5=-0.6289; p=0.0101). Blood glucose (β_5=-0.5561; p=0.0226) also inversely predicted *Th* expression, while plasma corticosterone (β_5=0.9238; p=0.0012) was a positive predictor of *Th* expression. The adjusted R^2 for this model was 0.9373 ($F_{4,5}$=18.7; p=0.0032). Prolactin additionally inversely predicted *Slc18a2* expression (**Table 7.6;** β_5=-0.2750; p=0.0191). Blood glucose (β_5=-0.3509; p=0.0106) also inversely predicted *Slc18a2* expression. Weight (β_5=0.2715; p=0.0482) and plasma corticosterone (β_5=0.6446; p=0.0003) were a positive predictors of *Slc18a2* expression. The adjusted R^2 for this model was 0.9588 ($F_{4,5}$=29.09; p=0.0012).

7.***3.5 Tyrosine hydroxylase, vesicular monoamine transporter-2, and dopamine transporter protein expression were unaffected by fructose-feeding in the dorsal striatum of both periadolescent and adult rats*** Differences in protein expression of the tyrosine hydroxylase (TH), and the vesicular monoamine transporter-2 (VMAT2), and the dopamine transporter (DAT) were examined in dorsal striatum of periadolescent and adult fructose- and chow-fed rats. TH did not differ between fructose-fed and chow-fed rats in both the periadolescent (**Figure 7.3a**; t_{11}=0.8372; p=0.4203) and adult (**Figure 7.3d**; t_{14}=0.1519; p=0.8814) cohorts. VMAT2 also did not differ between fructose-fed and chow-fed rats in both the periadolescent (**Figure 7.3b**; t_{12}=0.2582; p=0.8006) and adult (**Figure 7.3e**; t_{14}=0.2472; p=0.8084) cohorts. Finally, DAT did not differ between fructose-fed and chow-fed rats in both the periadolescent (**Figure 7.3c**; t_{14}=0.1021; p=0.9205) and adult (**Figure 7.3f**; t_{14}=0.2536; p=0.8035).

7.3.6 Periadolescent fructose-feeding did not alter tyrosine hydroxylase or vesicular monoamine transporter-2 protein expression in the hypothalamus, nucleus accumbens, and ventral tegmental area TH and VMAT2 protein expression were also examined in the hypothalamus, nucleus accumbens (NAcc), and ventral tegmental area (VTA) of periadolescent fructose-fed and chow-fed rats. It should be noted that this was a second cohort of rats used for metabolic analyses in Chapter Five as well as replication and validation experiments discussed in Chapter Six. Unlike the rats used for RNA-sequencing, these rats were not fasted or submitted to a saline challenge prior to euthanasia, and they were saline-perfused to prevent confounding of analysis with vascular factors.

In the hypothalamus, neither TH (**Figure 7.4a**; t_{12}=0.3730; p=0.7157) nor VMAT2 (**Figure 7.4d**; Kolmogorov-Smirnov D=0.4286, p=0.5752) expression differed between periadolescent fructose- and chow-fed rats. Similarly, neither TH (**Figure 7.4b**; t_{12}=0.7010; p=0.4967) nor VMAT2 (**Figure 7.4e**; t_{12}=q.247; p=0.2361) expression different between periadolescent fructose- and chow-fed rats in the VTA. Finally, neither TH (**Figure 7.4c**;

t_{12}=0.0868; p=0.9323) nor VMAT2 (**Figure 7.4f**; t_{12}=0.7070; p=0.4931) expression different between periadolescent fructose- and chow-fed rats in the NAcc.

7.4. Discussion

The current study highlighted the effect of periadolescent high-fructose feeding on expression of genes related to hypothalamic dopaminergic signaling without extension to mesolimbic circuitry. Based on principal component analysis of RNA sequencing, the dopamine transporter (DAT) and tyrosine hydroxylase (TH) were among ten highest-ranked genes in the top-loading factors for PC1, indicating their key role in defining variance in gene expression in the dataset of chow- and fructose-fed animals (**Table 7.2**). Correspondingly, differential expression analysis indicated that the high-fructose significantly altered hypothalamic expression of multiple genes related to dopamine synthesis and signaling, including the genes for the DAT, TH, dopamine receptors 1 and 2 (D1R and D2R), dopamine decarboxylase (DDC), and vesicular monoamine transporters 1 and 2 (VMAT1 and VMAT2; **Table 7.3**). In addition, though plasma prolactin was not significantly reduced in the fructose-fed cohort (p=0.1105), it was an inverse predictor of hypothalamic DAT, TH, and VMAT2 gene expression in chow- and fructose-fed animals (**Tables 7.4, 7.5, & 7.6**). However, protein expression of DAT, TH, and VMAT2 did not differ between chow- and fructose-fed animals in any brain region or cohort examined (**Figures 7.3 & 7.4**).

The altered expression of genes related to dopamine synthesis and signaling fits with literature indicating that hypothalamic dopamine is responsive to high-carbohydrate feeding and helps regulate satiety. The preference for carbohydrates in particular is mediated through its orosensory effects via gustatory reflex pathways, first through the pontine parabrachial nucleus and subsequently either through the thalamic taste area to the primary gustatory cortex, or widely throughout the ventral forebrain terminating in multiple regions including the hypothalamus and

amygdala (Hajnal & Norgren, 2005; Norgren, 1976). In response to food intake, the ventromedial nucleus of the hypothalamus (VMN) increases dopamine release after food intake, and release is associated with the duration of meal consumption (Meguid et al., 2000; Meguid, Yang, & Laviano, 1997).

In the present study, the increases in TH gene expression (**Table 7.3**), an intermediary precursor in dopamine synthesis, increases in VMAT1 and VMAT2, which package dopamine for subsequent release, and changes in D1R and D2R gene expression are consistent with evidence for altered hypothalamic dopaminergic turnover, though further experiments would be necessary to confirm the effect. Interestingly, the D2 receptor has been implicated in both compulsive-eating in rodents (P. M. Johnson & Kenny, 2010) as well as in clinical obesity (Carpenter, Wong, Li, Noble, & Heber, 2013). Decreased striatal D2R expression is has been linked to excessive adiposity in rats after compulsive eating of a cafeteria diet (P. M. Johnson & Kenny, 2010), though D2R KO mice have a lean phenotype and reduced consumption (Baik, 2013), perhaps indicating a role for the D2R in maintenance of energy homeostasis. Furthermore, the Taq1 A1 allele of the *DRD2* gene, which attenuates D2R expression in the dorsal striatum, has not only been associated with cocaine, alcohol, and opioid use, but is also associated with increased BMI (Carpenter et al., 2013). In the present study, however, we observed increased hypothalamic *Drd2* expression. The D2R has two isoforms due to alternative splicing, giving way to both a short and long form that act both pre- and post-synaptically. Only the post-synaptic isoform is known to be present in the tuberoinfundibular circuit (Baik, 2013). D2R agonists in the tuberoinfundibular circuit enhance dopamine metabolism (Berry & Gudelsky, 1991; Durham, Johnson, Eaton, Moore, & Lookingland, 1998), and activation of D2R has been implicated in hyperglycemia and insulin resistance (Garcia-Tornadu et al., 2010).

This potential for altered dopamine turnover is consistent with evidence for increases in the ventromedial nucleus dopamine release in response to feeding (Meguid et al., 2000), and increased basal dopamine in obese Zucker rats (Yang & Meguid, 1995). It should be noted,

however, that the end effect of feeding on dopamine in the across the entire hypothalamus remains controversial, as both anorectic and orexigenic effects have been observed depending on the site of increased dopamine, and the lateral hypothalamus and ventromedial nucleus have opposing dopaminergic responses to fasting and feeding (Legrand et al., 2015; Meguid et al., 2000). Moreover, while stimulation of the lateral hypothalamus (LHA) generally stimulates feeding and promotes a concomitant increase in accumbens dopamine (Hernandez & Hoebel, 1988), stimulation of LHA dopamine by injection of α-melanocyte stimulating hormone reduces food intake (Legrand et al., 2015). In this RNA-seq study, the whole hypothalamus was use, and thus regional discrimination in gene expression was not possible. However, the substantial change in gene expression indicates a profound effect of the high-fructose diet, and future studies will be necessary to determine the driving regional force.

As the available data in this study did not permit for direct measurement of dopamine release or turnover, we measured plasma prolactin as a proxy for hypothalamic dopamine. Prolactin, a polypeptide hormone secreted by lactotrophs of the anterior pituitary, is under dopaminergic regulation and dopamine is reported to be the most important prolactin-inhibiting factor found in the hypothalamus (Fitzgerald & Dinan, 2008). The gene for prolactin releasing hormone, *Prlhr,* was significantly downregulated in fructose-fed animals. Plasma prolactin, however, through reduced, was not significantly altered in fructose-fed animals (**Figure 7.2; p=0.1105**). However, given that samples with a CV of greater than 10% were removed from analysis, our final sample size included only five fructose-fed and six chow-fed animals, leaving us underpowered to detect significance with a power of only 0.35. Nonetheless, prolactin did predict hypothalamic gene expression of DAT, TH, and VMAT2 **(Tables 7.4, 7.5 & 7.6)**. This could potentially indicate a relationship between expression of these genes and altered hypothalamic dopamine, though future studies in a different cohort of animals examining dopamine release through techniques such as microdialysis or fast-scan cyclic voltammetry would be necessary.

Protein expression of DAT, TH, and VMAT2 was examined in the dorsal striatum of the same animals used for RNA-sequencing as well as a cohort of animals fed the high-fructose diet in adulthood only. None of these proteins differed in dorsal striatal expression between fructose- and chow-fed animals (**Figure 7.3**). In contrast to these findings, the dorsal striatum plays an important role in feeding, as selective restoration of dopamine in the dorsal striatum of dopamine-deficient mice is sufficient to reinstate feeding (Szczypka et al., 2001). However, mesolimbic circuitry across the VTA and nucleus accumbens, which interact with the hypothalamus, are better understood for their role in mediating hedonic responses to feeding (Avena, Bocarsly, et al., 2008; Shibata, Kameishi, Kondoh, & Torii, 2009). Chow (Schilstrom, Svensson, Svensson, & Nomikos, 1998), sucrose (Hajnal, Smith, & Norgren, 2004), and corn oil (Liang, Hajnal, & Norgren, 2006) all increase dopamine release and/or turnover in the nucleus accumbens, especially the accumbens shell. Consumption also regulates dopamine release in the ventral tegmental area (VTA;(Schilstrom et al., 1998)), and lesions of VTA dopaminergic neurons decrease preference for sucrose (Shibata et al., 2009). Hypothalamic nuclei can communicate with both the nucleus accumbens and the VTA through melanocortigenic and orexigenic nuclei, which may in turn alter dopamine turnover in these regions (Lutter & Nestler, 2009).

We therefore further examined protein expression of TH and VMAT2 in the hypothalamus, nucleus accumbens, and VTA. However, none of these regions showed any differences in protein expression (**Figure 7.4**). The animals used for these assays had not been fasted, unlike the animals used in the RNA-sequencing experiment. The majority of studies assessing differences in dopaminergic signaling in relation to feeding do so in a “challenge” condition, i.e., either immediately post-feeding (Avena, Bocarsly, et al., 2008; Hajnal et al., 2004; Shibata et al., 2009) or while fasting (Minano et al., 1989; Myers et al., 1988). Future experiments would be necessary to determine if the differences in protein expression in the hypothalamus did not follow gene expression changes due to this change in experimental protocol. In addition, future experiments during fasting and/or feeding would determine the

effect of these challenges on gene and protein expression in other regions, including the VTA and dorsal and ventral striatum.

In addition to the potential relationship to reward systems and addiction, the phenotype and findings in this study could lead to future novel studies in the field of attention deficit hyperactivity disorder (ADHD). Though a 1995 meta-analysis indicated no relationship between sugar consumption and ADHD (Wolraich, Wilson, & White, 1995), this relationship has again come under scrutiny due to the increased recognition of the role that dopamine signaling and alterations in dopamine gene expression play in both ADHD (Kirley et al., 2002; D. Li, Sham, Owen, & He, 2006) and in sugar consumption (R. J. Johnson et al., 2011). A similar downregulation of striatal dopamine receptors has been observed in both ADHD and drug addiction (R. J. Johnson et al., 2011). In addition, both children (Racicka, Hanc, Giertuga, Brynska, & Wolanczyk, 2015) and adults with persistent or remittent ADHD (Cortese, Faraone, Bernardi, Wang, & Blanco, 2013) suffer from greater rates of obesity and overweight that unaffected populations. Johnson et al (R. J. Johnson et al., 2011) have proposed that chronic sugar consumption could lead to intermittent excessive dopamine stimulation, resulting in downregulation of striatal dopamine receptors and a compensatory continued consumption of sugar. The subsequent relative dopamine depletion results in loss of prefrontal inhibition, linking to both compulsive eating and ADHD. While this hypothesis remains to be tested and the role of hypothalamic dopamine that moderates this mesolimbic reponse must be clarified, it remains an interesting area for future study.

In conclusion, the current study demonstrates the utility of combining biostatistical analyses to generate hypotheses and drive experimental design. The data presented provide evidence that, in the fasting state, male rats fed a high-fructose diet throughout adolescence differ from chow-fed rats in hypothalamic expression of multiple genes related to dopamine synthesis and signaling. Subsequent research sought to determine whether gene expression changes translated into either altered protein expression or changes in dopamine-dependent hormonal

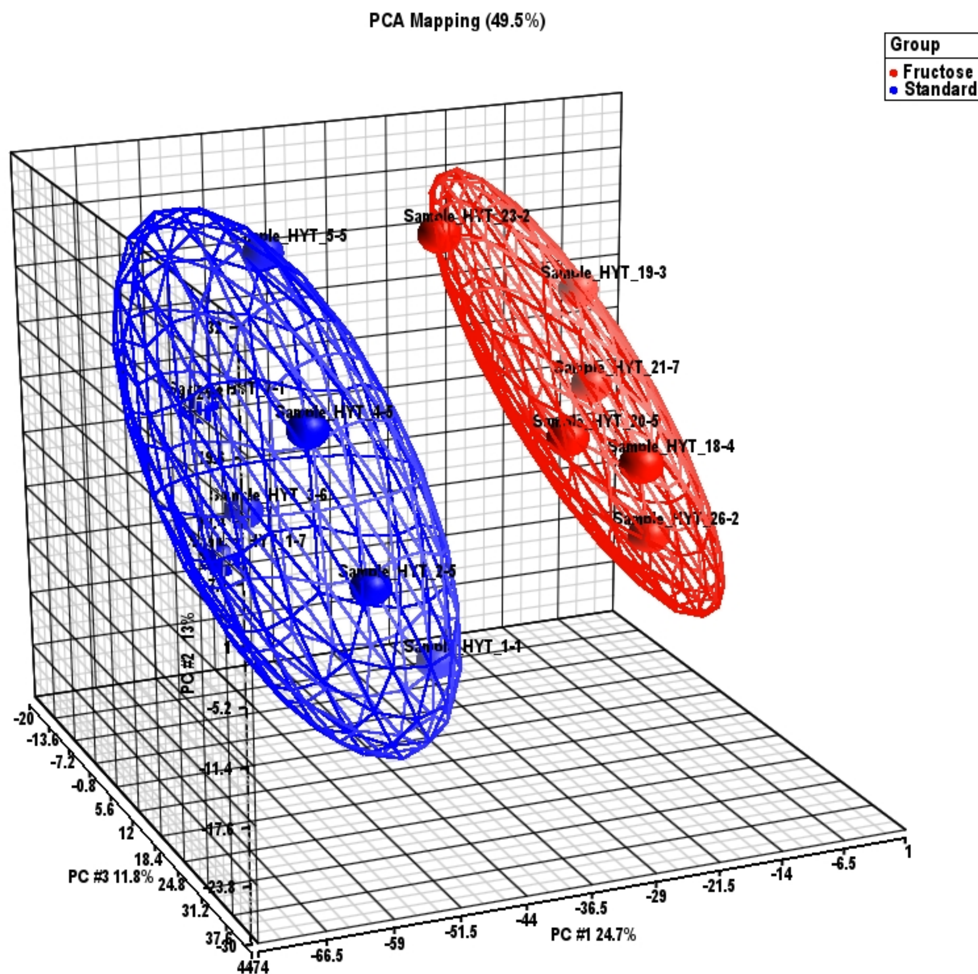
state. Results indicated that plasma prolactin, under inhibitory control from hypothalamic dopamine, predicted expression of dopamine-related genes in fructose-fed rats. However, it remains unclear whether these changes extend to protein expression or functional changes in dopamine release and activity. Future research will additionally need to clarify whether the differences between chow- and fructose-fed animals are unique to the fasting state, or if changes in dopaminergic gene expression after consumption of a periadolescent high-fructose diet are indeed confined to the hypothalamus. Understanding these effects will shed light on the role of diet and hypothalamic dopamine in particular in modulating homeostatic mechanisms (regulating energy storage and satiety) as well as hedonic circuitry (regulating reward responses to food).

Table 7.1.

Cohort	Age	Diet	Stress	Experimental Use	N
Cohort 1	Periadolescent	Chow	Non-Stress	RNA-seq, Prolactin ELISA Rapidly Decapitated after Fasting and Saline Injection for Glucose Tolerance Test	7
		Fructose			6
Cohort 2		Chow	Non-Stress	Western Blotting (Dopamine Transporter, Tyrosine Hydroxylase, Vesicular Monoamine Transporter 2) Saline-Perfused, Non-Fasting	8
		Fructose			10
Adult Cohort	Adult	Chow	Non-Stress	Western Blotting (Dopamine Transporter, Tyrosine Hydroxylase, Vesicular Monoamine Transporter 2) Rapidly Decapitated, Non-Fasting	8
		Fructose			8

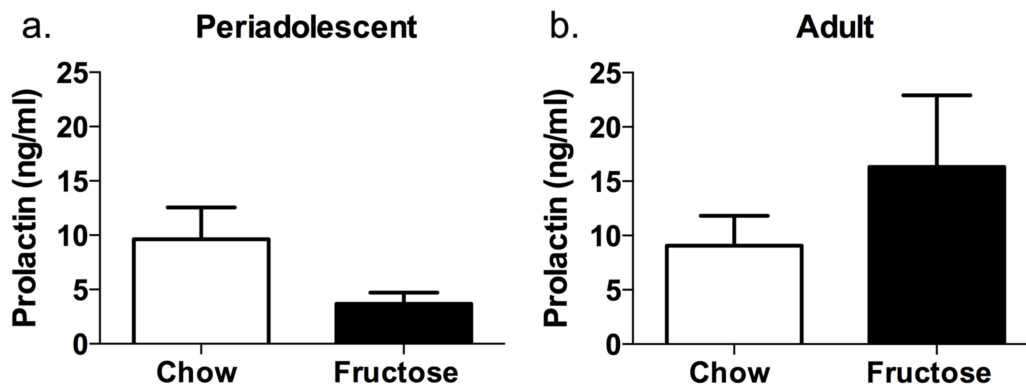
Cohort Distribution and Use. This lists the number and use of all animals in Chapter 7. For a full description of all uses of each cohort, see Chapter Five.

Figure 7.1.



Principal component analysis demonstrates separation of hypothalamic gene expression based on diet type Principal component analysis (PCA) was used to provide another means of assessing genes that drive the differences between chow-fed and fructose-fed animals. PCA identified two distinct populations, perfectly clustered by diet type. Principal component 1 (PC1) accounted for 24.7% of the variance in gene expression, while PC2 accounted for 13.0% and PC3 accounted for 11.8% of the variance, collectively accounting for 49.5% of the total variance in gene expression. Chow-fed animals are represented in blue, while fructose-fed animals are shown in red.

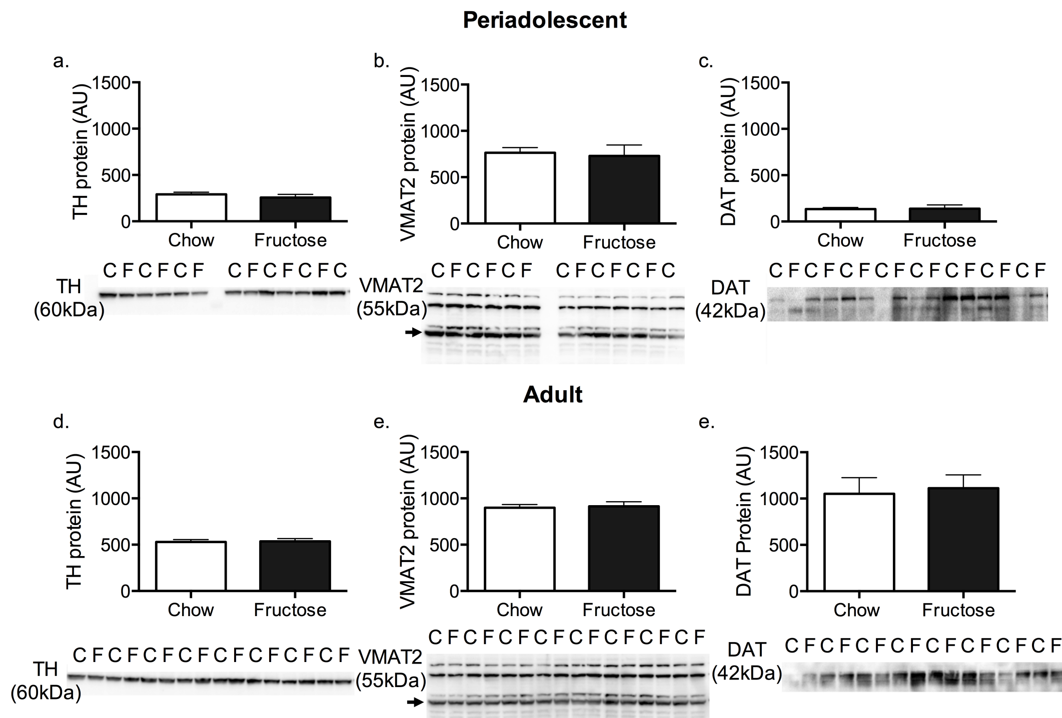
Figure 7.2.



Plasma prolactin is not significantly altered in either periadolescent or adult fructose-fed rats

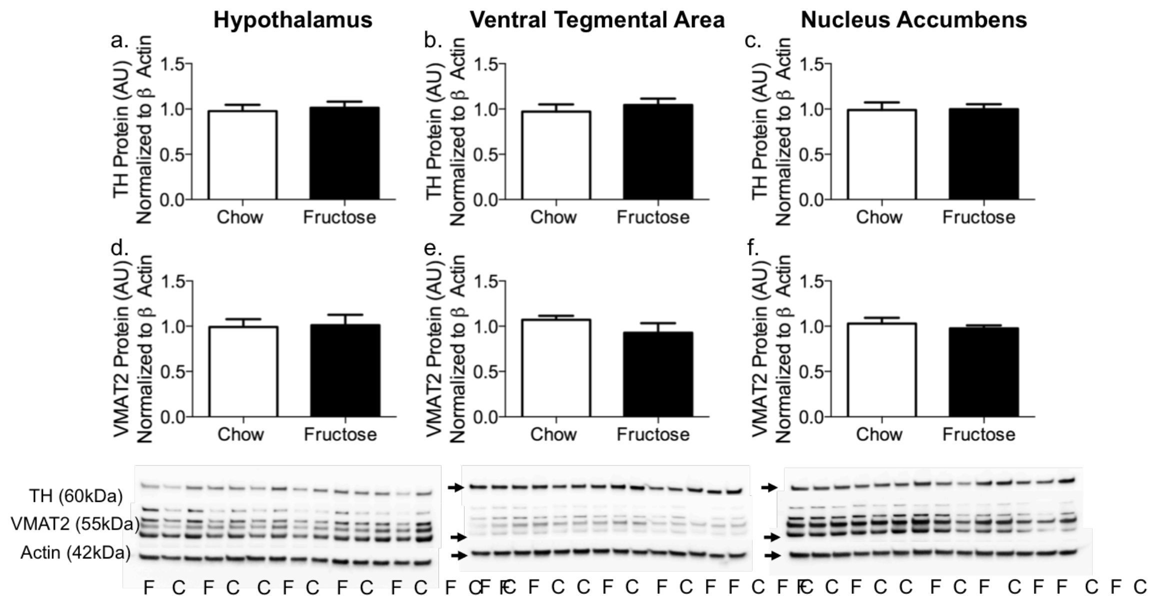
a. Though diminished, plasma prolactin was not significantly decreased in periadolescent fructose-fed rats. b. Plasma prolactin was also not significantly changed in adult fructose-fed rats, though it was slightly increased relative to chow fed rats. Data shown are mean ± SEM.

Figure 7.3



Tyrosine hydroxylase, vesicular monoamine transporter-2, and dopamine transporter protein expression were unaffected by fructose-feeding in the dorsal striatum of both periadolescent and adult rats Differences in protein expression of the tyrosine hydroxylase (TH), and the vesicular monoamine transporter-2 (VMAT2), and the dopamine transporter (DAT) were examined in dorsal striatum of periadolescent and adult fructose- and chow-fed rats. **a,b,c**. None of these proteins differed in expression in the dorsal striatum of either periadolescent or adult animals. Representative blots of each protein are underneath the respective graph. Data shown are mean ± SEM.

Figure 7.4



Periadolescent fructose-feeding did not alter tyrosine hydroxylase or vesicular monoamine transporter-2 protein expression in the hypothalamus, nucleus accumbens, and ventral tegmental area TH and VMAT2 protein expression were also examined in the hypothalamus, nucleus accumbens (NAcc), and ventral tegmental area (VTA) of periadolescent fructose-fed and chow-fed rats. There were no differences in protein expression between fructose and chow-fed animals in any brain regions. Representative graphs are underneath their respective graphs. Data shown are mean ± SEM.

Table 7.2. Loading factors from Principal Component Analysis of Hypothalamic RNA sequencing in Chow-Fed and Fructose-Fed Rats

Top Loading					
PC1	Loading	PC2	Loading	PC3	Loading
Slc6a3	0.142	*Slc6a3*	0.108	*Chrnb3*	0.098
Chrnb3	0.102	*Gata3*	0.079	*Slc6a3*	0.091
Chrna6	0.089	*Chrna5*	0.071	*Chrna5*	0.062
Chrna5	0.079	*Pla2g16*	0.066	*Smyd1*	0.060
Slc39a4	0.075	*RT1-Ba*	0.065	*RT1-Da*	0.058
Mas1	0.063	*Slc39a4*	0.059	*Cpz*	0.058
Slc10a4	0.057	*LOC689064*	0.057	*Chrna6*	0.055
Th	0.056	*Alas2*	0.055	*Hspb7*	0.055
Dennd2d	0.049	*Fut7*	0.054	*Sntb2*	0.054
Dmrta2	0.048	*Gprc5a*	0.054	*Ttc29*	0.053

Bottom Loading					
PC1	Loading	PC2	Loading	PC3	Loading
Chst9	-0.063	*Npbwr1*	-0.052	*Fam150b*	-0.047
Synpo2	-0.063	*Otop1*	-0.052	*Exo1*	-0.048
Adra2b	-0.065	*Otp*	-0.053	*Meis2*	-0.049
Shox2	-0.075	*Trh*	-0.054	*Nox4*	-0.053
Vip	-0.077	*Prlhr*	-0.055	*Trh*	-0.055
Smpx	-0.083	*Zap70*	-0.057	*Foxg1*	-0.059
Gabrd	-0.085	*Tbr1*	-0.061	*Mybl2*	-0.065
Prkcd	-0.087	*Fezf1*	-0.106	*Fezf1*	-0.068
Mybpc1	-0.091	*Oxt*	-0.140	*Oxt*	-0.160
Wnt9b	-0.095	*Avp*	-0.152	*Avp*	-0.174

Table 7.3.

Name	**Gene**	**Chow FPKM**	**Fructose FPKM**	**log_2 (FPKM)**	**Fold Change**	**Test Statistic**	**p value**	**q value**
Tyrosine	*Tyr*	0.0798	0.1067	0.4192	1.3372	0.0000	1.0000	1.0000
Tyrosine hydroxylase	*Th*	22.1102	124.713	2.4958	5.6405	11.8471	0.0001	0.0010 *
Dopamine decarboxylase	*Ddc*	6.5237	16.8715	1.3708	2.5862	5.6514	0.0001	0.0010 *
Dopamine beta hydroxylase	*Dbh*	0.0249	0.0576	1.2110	2.3149	0.0000	1.0000	1.0000
Vesicular monoamine transporter-1	*Slc18a1*	1.3337	2.1812	0.7097	1.6355	1.4804	0.0010	0.0155 *
Vesicular monoamine transporter-2	*Slc18a2*	9.6833	29.8498	1.6242	3.0826	7.1239	<0.0001	0.0010 *
Dopamine receptor-1	*Drd1*	4.0301	2.7247	-0.5647	0.6761	-1.4805	0.0005	0.0083 *
Dopamine receptor-2	*Drd2*	7.7724	15.0269	0.9511	1.9334	3.7524	0.0001	0.0010 *
Dopamine receptor-3	*Drd3*	0.3841	0.5725	0.5757	1.4904	0.0000	1.0000	1.0000
Dopamine receptor-4	*Drd4*	0.0482	0.1247	1.3713	2.5870	0.0000	1.0000	1.0000
Dopamine receptor-5	*Drd5*	2.0326	2.3324	0.1985	1.1475	0.3929	0.3853	0.7939
Dopamine transporter	*Slc6a3*	1.3562	63.3712	5.5462	46.7265	12.2134	0.0001	0.0010 *
Monoamine oxidase-a	*Maoa*	38.3168	39.0482	0.0273	1.0191	0.1347	0.7548	0.9565
Monoamine oxidase-b	*Maob*	38.6801	45.1574	0.2234	1.1675	1.1239	0.0083	0.0809
Catechol-O-methyl transferase	*Comt*	48.4790	49.4233	0.0278	1.0195	0.1111	0.7773	0.9610
Phenylethanolamine n-methyltransferase	*Pnmt*	0.0497	0.1185	1.2531	2.3835	0.0000	1.0000	1.0000

Dopamine Synthesis and Signaling Related Genes: Relative Hypothalamic Expression between Chow- and Fructose-Fed Animals. The relative expression in log_2(FPKM) as well as test statistic and q value after multiple testing corrections are shown for several key genes related to dopamine signaling.

Table 7.4.

Predictor of *Slc6a3*	**Beta**	**Error**	**t value**	**p value**
Intercept	2.7496	0.4988	5.513	0.0023*
Prolactin	-1.9848	0.5776	-3.436	0.0185*
Weight	2.0068	0.7469	2.687	0.0435*
Blood Glucose	-1.4932	0.6312	-2.366	0.0643
Corticosterone	2.4445	0.5153	4.744	0.0051*

Adjusted R^2=0.7982; $F_{4,5}$=9.902; p=0.0135; AIC=41.07575

Plasma prolactin predicts dopamine transporter expression in RNA-sequencing. Decreased prolactin and weight as well as increased corticosterone predict increased dopamine transporter expression in RNA-sequencing in chow and fructose-fed periadolescent rats.

Table 7.5.

Predictor of *Th*	Beta	Error	t value	p value
Intercept	5.5642	0.1350	41.201	<0.0001*
Prolactin	-0.6289	0.1564	-4.021	0.0101*
Weight	0.4937	0.2022	2.441	0.0586
Blood Glucose	-0.5561	0.1709	-3.254	0.0226*
Corticosterone	0.9238	0.1395	6.621	0.0012*

Adjusted R^2=0.9373; $F_{4,5}$=18.7; p=0.0032; AIC=14.9446

Plasma prolactin predicts tyrosine hydroxylase expression in RNA-sequencing Decreased prolactin and blood glucose as well as increased weight and corticosterone predict increased tyrosine hydroxylase expression in RNA-sequencing in chow and fructose-fed periadolescent rats.

Table 7.6.

Predictor of *Slc18a2*	Beta	Error	t value	p value
Intercept	3.9483	0.0697	56.637	<0.0001*
Prolactin	-0.2750	0.0807	-3.407	0.0191*
Weight	0.2715	0.1044	2.601	0.0482*
Blood Glucose	-0.3509	0.0882	-3.977	0.0106*
Corticosterone	0.6446	0.0720	8.950	0.0003*

Adjusted R^2=0.9588; $F_{4,5}$=29.09; p=0.0012; AIC=1.7196

Plasma prolactin predicts vesicular monoamine transporter-2 expression in RNA-sequencing

Decreased prolactin and blood glucose as well as increased weight and corticosterone predict increased vesicular monoamine transporter-2 expression in RNA-sequencing in chow and fructose-fed periadolescent rats.

Chapter Eight:

High-fructose diet initiated during adolescent development alters cerebral vasculature but does not exacerbate behavioral outcomes after middle cerebral artery occlusion.

Adapted from:

Harrell, CS, Zainaldin, C, Vaughan, L, McFarlane, D, Stein, D, Sayeed, I, Neigh, GN. High-fructose diet during adolescent development alters cerebral vasculature but does not exacerbate behavioral outcomes after middle cerebral artery occlusion. *In Preparation.*

8.0 Abstract

Depression affects one third of stroke survivors, resulting in impaired recovery and increased mortality. Current literature links post-stroke depression to ischemia-induced injury and associated changes in endothelial function, inflammation and neural circuitry. This ischemic injury may come as a "second hit" to pre-existing metabolic pathology that is itself associated with depression, such as diabetes and metabolic syndrome. This study takes advantage of a periadolescent high-fructose diet model to determine whether diet-induced metabolic dysfunction exacerbates behavioral outcomes or lesion volume after middle cerebral artery occlusion. We demonstrate that high-fructose diet consumed during adolescent development increased vascularization of region CA1 of the hippocampus, elevated baseline plasma corticosterone, and promoted anxiety-like behavior. However, periadolescent fructose consumption did not exacerbate neurological behaviors or lesion volume after middle cerebral artery occlusion, potentially due to adaptive cerebrovascular responses to the metabolic influence of the high-fructose diet. Future studies will be necessary to assess additional modifiable factors that affect risk for post-stroke depression.

8.1 Introduction:

Depression affects one third of stroke survivors (Hackett, Yapa, Parag, & Anderson, 2005) and results in impaired recovery (Gainotti, Antonucci, Marra, & Paolucci, 2001) and increased mortality (House, Knapp, Bamford, & Vail, 2001). This post-stroke depression causes not only human suffering but also greater burden to an already over-burdened healthcare system, increasing both inpatient and outpatient stays up to three years post-stroke (Ghose, Williams, & Swindle, 2005). Research on post-stroke depression provides an opportunity to alleviate this burden and shed light on the mechanisms underlying the neurobiology of depression (Alexopoulos, 2006; Whyte, Mulsant, Vanderbilt, Dodge, & Ganguli, 2004), since symptoms occur more frequently after stroke than after similar debilitating disease (Folstein, Maiberger, & McHugh, 1977).

The underlying neurobiology of post-stroke depression likely differs from that of depression not associated with stroke, and is likely related to ischemia-induced injury and associated changes in endothelial function, inflammation and neural circuitry (Whyte et al., 2004). This ischemic injury may come as a "second hit" to pre-existing metabolic pathology that is itself associated with depression, such as diabetes and metabolic syndrome (R. J. Anderson et al., 2001; Lamers et al., 2013). Such metabolic pathology is a known risk factor for post-stroke depression, particularly in young adults (Tanislav et al., 2015), which is relevant given that diabetes and obesity affects a growing proportion of children (Nadeau & Dabelea, 2008; Ogden et al., 2014). This leaves an increasing population in a state susceptible not only to additional energetic stressors such as stroke (Gunnell, Frankel, Nanchahal, Peters, & Davey Smith, 1998; Reilly & Kelly, 2011), but also to increased risk of post-stroke depression (Tanislav et al., 2015). Thus, understanding the unique etiology and risk factors for post-stroke depression is of growing importance.

Animal models have elicited several possible mechanisms that may drive vulnerability to depressive behavior after a vascular event such as stroke. Ischemic events alter the hypothalamic-pituitary-adrenal (HPA) axis response to acute stress (Nemeth & Neigh, 2015), and exposure to stress after ischemia can induce depressive-like behavior (Wang, Zhang, Guo, Teng, & Chen, 2008). Glucocorticoid-mediated changes in cerebrovasculature may partially underlie these behavioral changes. Exposure to exogenously administered glucocorticoids in adulthood inhibits cerebral angiogenesis (Ekstrand, Hellsten, Wennstrom, & Tingstrom, 2008) and increases microvascular endothelial cell death (Vogt & Schmid-Schonbein, 2001). Adult stress also reduces vasculature in the hippocampus, a region known to regulate affective behavior (B. S. McEwen & Magarinos, 2001), and these effects are exacerbated by prenatal glucocorticoid exposure (Neigh et al., 2009).

In this study, we hypothesized that exposure to a high-fructose diet could remodel hippocampal cerebrovasculature and precipitate anxiety- and depressive-like behavior after an ischemic event. In Chapter Five, we demonstrated that a high-fructose diet consumed during adolescence results in negative metabolic outcomes in adulthood, remodeling of the HPA axis, and depressive- and anxiety-like behaviors. Others have additionally recapitulated these effects on insulin resistance, blood pressure, and dyslipidemia(Catena et al., 2003; Hwang et al., 1987; Kasim-Karakas, Vriend, Almario, Chow, & Goodman, 1996), as well as on the hypothalamic-pituitary-adrenal (HPA) axis and peripheral glucocorticoid levels (Brindley et al., 1981; Kinote et al., 2012). The 25% increase in fructose consumption since the 1970s accounts for part of the dramatic rise in obesity and insulin resistance (Elliott et al., 2002), and adolescents are the highest consumers of fructose, with over 25% of adolescents receiving 15% or more of daily calories from fructose alone (Vos et al., 2008). Understanding the role of fructose in cerebrovascular remodeling and vascular depression can help drive further research and policy.

Here, we exposed male rats to either high-fructose or standard chow from weaning until adulthood. In one cohort, also used in Chapters Five through Seven, we determined whether the

diet altered hippocampal expression of key vascular and glucocorticoid receptor-related genes, cerebrovasculature and blood-brain-barrier permeability. In a second cohort, we determined whether the diet exacerbated the effects of middle cerebral artery occlusion on neurologic and affective behavior; the hormonal response to stress; and lesion volume.

8.2 Materials and Methods:

8.2.1 Animal Husbandry: Timed pregnant Wistar rats (n=12) were obtained on gestational day 12 from Charles River (Wilmington, MA). Rats were housed on a 14:10 reverse light: dark cycle in a facility controlled for humidity (60%) and temperature (20 °C-23 °C). Litters were culled on postnatal day (PND) 3 to eight pups per litter and males were selected and weaned on PND 23 (n=84). Of these animals, one cohort was used for baseline cerebrovascular assessments without surgery, and will henceforth be referred to as the "Vascular cohort" (Chow n=16, Fructose n=20). Two cohorts were used for assessment of outcomes after sham surgery or MCAO surgery (Chow-Sham=12, Chow-MCAO=11, Fructose-Sham=12, Fructose-MCAO=10). These cohorts will henceforth be referred to as "Surgery cohort 1" and "Surgery cohort 2." Descriptions of each cohort, exact n per cohort, and use are clarified in **Table 8.1**. The Vascular Cohort in this Chapter is the same as the Metabolic cohort in Chapter Five (**see Table 5.**1). Both Vascular and Surgery cohorts were used in the assessment of diet-induced metabolic effects prior to surgery. Animals were reared in-house and pair-housed until the MCAO or sham surgery, at which time animals were single-housed thereafter through the end of experimentation and euthanasia. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Emory University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

8.2.2 Diet: Two days post-weaning, male rats were assigned to one of two diet groups: either standard chow (Chow; n=40; Lab Diets Rodent Diet 5001), or the high-fructose diet

(Fructose; n=44; Research Diets D05111802; see **Table 8.1** for breakdown of n by cohort). The fructose diet used was 55% energy fructose and the standard chow used was 0.30% energy fructose. Both diets used contained comparable levels of vitamins and minerals deemed necessary for rodent health, and were reviewed by veterinary staff and approved by IACUC. Dietary composition of this diet is described in **Table 5.2**. Diets containing a similar percentage of fructose can induce cardiac remodeling (Chess, Lei, Hoit, Azimzadeh, & Stanley, 2007), increase mortality in the context of hypertension (N. Sharma et al., 2007), and cause leptin resistance (Haring & Harris, 2011). In Chapter Five, we additionally showed that this diet, when consumed during adolescence, induces remodeling of the hypothalamic transcriptome and increases depressive- and anxiety-like behavior in adulthood.

In these experiments, all cohorts consumed either the high-fructose diet from PND25 throughout adolescent development and into adulthood (terminal euthanasia at PND85-100). While adolescence is difficult to define precisely in rats as in humans, it is accepted that infancy and "childhood" end at weaning (PND21-23) and that adulthood begins at PND60 (McCormick & Mathews, 2007; Spear, 2000). Surgical, behavioral, and terminal endpoints for were selected to allow these experiments to be performed in adulthood, given data from Chapter Five demonstrating metabolic and behavior abnormalities in adulthood. The experimental timelines for each cohort are displayed in **Figure 8.1**.

8.2.3 Metabolic Assessments: Metabolic assessments were taken from both Surgery cohorts for the first six weeks on the diet prior to behavioral testing and surgery. As previously described in Chapter Five, blood glucose was tested weekly after an overnight fast by tail prick using a *Freestyle* glucometer. Animal weights were also taken concurrently with glucose readings. Research assistants, carefully accounting for any spilled food, measured food consumption daily and caloric consumption was determined thereof. To determine caloric efficiency, the body mass gained per week per animal was divided by the mean weekly caloric consumption calculated per cage (of pair-housed animals) divided by two. While imprecise, this

type of approximation should only serve to increase variability in caloric efficiency and thus increase probability of returning a false negative result as opposed to producing a false positive. These metrics were also measured for the Vascular cohort, but have been previously discussed in Chapter Five. Here, only terminal weights were taken for the Vascular cohort are reported and discussed. Pre-surgery, post-surgery, and terminal weights were taken for the Surgical cohorts.

8.2.4 Corticosterone Analyses: Blood was collected for corticosterone analyses from the following cohorts at the following time points: Vascular cohort, PND89-90, trunk blood; Surgery Cohort 1: PND78-baseline, PND 83-post-FST, tail snip; and Surgery Cohort 2: PND94-baseline, PND 99-post-FST, tail snip. All animals were allowed to acclimate to the testing room for two hours before handling, and all collections occurred in a separate room from the testing room. Tail snips were performed on all animals within two minutes to allow collection prior to the snip stress-induced elevation of corticosterone. For the trunk blood, a subset of the Vascular cohort animals (fructose: n=10; chow: n=8; see **Table 8.1**) was euthanized with Euthasol® after nine weeks on diet, the chest cavity opened, and trunk blood immediately collected prior to perfusion with saline for two minutes. Blood was collected in EDTA coated tubes and spun at 5400 rcf (3000 rpm in a Sorvall SM-24 rotor) for 20 minutes for plasma collection. Tail vein blood was collected in uncoated tubes and spun at 5000 rcf (Eppendorf 5415R Centrifuge) for 20 minutes for serum collection. Corticosterone was measured in both plasma and serum via ELISA (sensitivity 27 pg/mL, Enzo Life Sciences, Farmingdale, NY, USA). All samples were run in duplicate.

8.2.5 Quantitative RT-PCR As just described, after nine weeks on diet at PND89-90, a subset of the Vascular cohort animals (fructose: n=10; chow: n=8) was euthanized with Euthasol® and perfused with saline for two minutes, trunk blood was collected, adrenals and testes were collected and weighed, and brains were flash frozen before storage at -80^{oC}. To determine whether the fructose diet had an effect on a marker of hippocampal angiogenic-related gene expression, we examined expression of vascular endothelial growth factor a (*Vegfa*). The left

hippocampus was lysed and homogenized using Trizol RNA Extraction reagent (Life Technologies, Grand Island, NY). RNA was then extracted with an RNEasy kit from Qiagen, and integrity was assessed with a NanoDrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE). RNA was standardized based on the NanoDrop 2000 readings and then reverse-transcribed using the High Capacity RNA to cDNA kit (Life Technologies, Grand Island, NY). cDNA was quantified with the PicoGreen Assay (Invitrogen, Carlsbad, CA) and then standardized to 10 pg/µl. Rat TaqMan Gene Expression Assays were purchased from Life Technologies (Grand Island, NY) with probes labeled with 6-FAM and MGB (non-fluorescent quencher) at the 5' and 3' ends, respectively: *Vegfa* (Rn01511601_m1) and *Hprt1* (Rn01527840_m1).

The following two-step RT-PCR cycling conditions were used on the 7900HT Sequence Detection System (Applied Biosystems): 50° C (2 min), 95° C (10 min), 40 cycles of 95° C (15 s) and 60° C (1 min). The housekeeping gene *Hprt1* was run as an endogenous control. Relative gene expression of individual samples run in triplicate (with coefficient of variation cut-off set to 4%) was determined by the comparative $\Delta\Delta C_T$ quantification method with fold change to standard chow of a given developmental cohort. All TaqMan gene expression assays are guaranteed to have 90 - 100% amplification efficiency as determined by the genome-aided probe and primer design pipeline and reported in the "Amplification Efficiency of TaqMan Gene Expression Assays" Application Note 127AP05-03 from Life Technologies.

8.2.6 Blood-Brain-Barrier Permeability A subset of the Vascular cohort (chow, n=8; fructose, n=10; see **Table 8.1**) were designated for immunohistochemical assessment if cerebrovascular remodeling. These animals were euthanized on PND89 with Euthasol® perfused for two minutes with saline and ten minutes with 4% paraformaldehyde for ten minutes. Brains were removed and post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and sectioned at 40 µm on a cryostat. In addition, adrenals and testes were collected and weighed.

Sections encompassing the entire rostro-caudal axis of the brain from vascular cohort brains (chow, n=8 and fructose, n=10) were stained for IgG (8-12 sections per animal, section sampling fraction (ssf) = 1/12). After blocking, sections were incubated in goat anti-rat IgG (1:1000, BA9400, Vector Labs, Burlingame, CA) overnight at 4^{oC}, washed, incubated with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA), and visualized with diaminobenzidine (SigmaFast 3,3'diaminobenzidine tablets, Sigma Aldrich, St. Louis, MO). Increased cerebral IgG presence indicates increased blood-brain-barrier (BBB) permeability, as endothelial cell tight junctions typically prevent transport of large molecules such as IgG across the BBB(Abbott, Patabendige, Dolman, Yusof, & Begley, 2010). A Nikon Eclipse 90i microscope (Melville, NY) fitted with MicroBrightField Stereoinvestigator Version 11 (MBF Bioscience, Williston, VT) was used to visualize IgG. To assess permeability, a blinded research assistant captured images of each brain section using Stereoinvestigator and calculated the percentage of the brain immunoreactive for IgG using the Gray Level Index to quantify mean optical density for each brain (ImageJ, NIH).

8.2.7 Vascular Length: Alternate sections from the staining above were used to assess changes in cerebral vasculature. Sections (5-6 sections per animal, ssf=1/12) encompassing the hippocampus from both chow (n=6) and fructose (n=6) were stained for rat endothelial cell antigen (mouse anti-RECA; MCA970R, ABD Serotec, Raleigh, NC). Sections (5-6 sections per animal, ssf=1/12) encompassing the amygdala from both chow (n=3) and fructose (n=3) were stained for RECA as well. After blocking, sections were incubated in mouse-anti RECA (1:200) overnight at 4^{oC}, washed, incubated with biotinylated horse anti-mouse (1:200, BA2000, Vector Labs, Burlingame, CA), washed, incubated with streptavidin peroxidase (1:200, S2438, Sigma Aldrich, St. Louis, MO), and visualized with diaminobenzidine (SigmaFast 3,3'diaminobenzidine tablets, Sigma Aldrich, St. Louis, MO). Vascular length was assessed in the hippocampus and its subregions cornu ammonis 1 (CA1), CA2, CA3, and the dentate gyrus, as well as in the amygdala. All assessments were made by a blinded research assistant using the Spaceballs probe

from Stereoinvestigator (MBF Bioscience, Williston, VT) on the same Nikon Eclipse 90i microscope (Melville, NY) described above.

8.2.8 Surgery: In adulthood, at either PND 65-66 (Surgery Cohort 1, Chow n=12, Fructose n=11) or PND 81-82 (Surgery Cohort 2, Chow n=11, Fructose n=11), half of each diet group was submitted to transient (90 minute) middle cerebral artery occlusion (MCAO; n=5-6 per diet per time point) as previously described (Yousuf, Atif, Sayeed, Tang, & Stein, 2014; Yousuf et al., 2015). The other half of each diet group in each cohort underwent sham surgery at the same time (n=6 per diet per time point). In brief, the MCAO surgery proceeded as follows: a midline incision was made on the ventral surface of the rat's neck, and the right common carotid artery was isolated and ligated with 6.0 suture. The internal carotid and pterygopalatine arteries were occluded with a microvascular clip. A 4.0 filament was introduced through the external carotid artery into the internal carotid artery and advanced approximately 20mm distal to the carotid bifurcation. Relative cerebral blood flow (CBF) was monitored by laser Doppler flowmetry for the entire 90 minutes of occlusion. After this time, the occluding filament was withdrawn to allow for reperfusion. Relative CBF was monitored before the wound was sutured and the rats were permitted to recover from anesthesia. The percent occlusion was calculated from CBF as Heartbeat and blood oxygen saturation levels were also monitored during surgery using a SurgiVet pulse oximeter (SurgiVetTM model V3304, Waukesha,WI, USA). Three rats died either during the MCAO surgery or in the post-MCAO period; one fructose-fed rat in cohort 1, and one fructose-fed and one chow-fed rat in cohort 2, as noted in **Table 8.1.**

8.2.9 Neurologic Behavior Assessment: Prior to surgery (PND58 for Surgery cohort 1; PND68 for Surgery cohort 2), rats were trained on four different tests to assess neurologic behavior: open field during the dark cycle to measure spontaneous locomotor activity(Whishaw & Kolb, 2004); sticky dot to measure somatosensory neglect(Whishaw & Kolb, 2004); grip strength to assess force capacity to pull a grid assembly(Atif et al., 2013; Yousuf et al., 2015); and rotorod testing to assess motor coordination (Atif et al., 2013; Yousuf et al., 2015). Rats

were tested on each assessment prior to surgery and again 72 hours post-surgery as previously described (Atif et al., 2013; Yousuf et al., 2015). Brief descriptions of each test follow. *Dark cycle open field:* Both pre- and post-injury, rats underwent a five-minute open field test during their dark cycle, in which overall locomotor activity in a 75cm x 75cm box was measured. Behavior was recorded by a video camera that was connected to an automated behavior analysis system (Capture Star, CleverSys, Inc, Reston, VA, USA). A blinded research assistant subsequently analyzed behavior on related automatic behavioral analysis systems (TopScan and Forced Swim, CleverSys, Inc, Reston, VA, USA). *Sticky Dot:* To determine somatosensory asymmetry, each rat will receive adhesive stimuli (one hemisphere of a round sticky dot of 12 mm in diameter) both pre- and post-injury attached to the distal radial aspect of the left forelimb during the animal's dark cycle. The time taken for the animal to remove the label is recorded during a 60-second observation period. Three trials per animal at each time-point were averaged for analysis at both time points (Atif et al., 2013; Whishaw & Kolb, 2004). *Grip Strength:* Animals were evaluated for the degree of force necessary to make the animal release a pull grid assembly by the forepaws using a grip strength meter. Rats were acclimated to the grip strength meter pre-injury for two days, pulling the grid assembly three times each day. On the third day, rats again pulled the assembly three times, and these measures were averaged and used as baseline measures. The test was repeated three days after surgery. *Rotorod:* Pre-injury, rats were trained on the rotorod apparatus to stay on the accelerating rod for 120 seconds. The animals will first be habituated to the stationary rod. After habituation they were exposed to the rotating rod. The rod will be started at 2 rpm and accelerated linearly to 20 rpm within 300 sec. The rats were trained for three to five days pre-surgery depending on their individual latency to stay on the accelerating rod for 120 seconds. A test trial was then performed, in which latency to fall off the rotorod was calculated for analysis. The test trial was repeated three times per animal. This same test was also performed three days post-surgery. The two best (largest) fall latency values a rat

achieved were then averaged and used for data analysis. Rats not falling off within 5 min will be given a maximum score of 300 seconds (Atif et al., 2013; Yousuf et al., 2015).

8.2.10 Affective Behavior Assessment: Affective behavior assessment was performed two weeks after surgery (PND79 for Surgery cohort 1 and PND95 for Surgery cohort 2). This time point was selected due to prior evidence from our lab indicating expression of depressive- and affective-type manifests two-weeks following microembolism-induced ischemia (Nemeth, Shurte, McTigue, Nemeroff, & Neigh, 2012). The tests used in this assessment were, in order of testing, open field during the light cycle to test locomotor activity and anxiety-like behavior (Lynn & Brown, 2010; Prut & Belzung, 2003); social interaction to examine anxiety-like and anhedonic behavior (File & Hyde, 1978); the elevated plus maze, to examine anxiety-like behavior (Pellow et al., 1985); and the forced swim test, originally designed to measure antidepressant efficacy, which can be used to assess depressive-like behavior (Porsolt et al., 1977).

Behavioral assessments were performed as previously described (Cite US, Nemeth). All behaviors were recorded by a video camera that was connected to an automated behavior analysis system (Capture Star, CleverSys, Inc, Reston, VA, USA). A blinded research assistant subsequently analyzed behavior on related automatic behavioral analysis systems (TopScan and Forced Swim, CleverSys, Inc, Reston, VA, USA). Details of individual assessments are briefly described here. *Light Cycle Open Field:* Rats underwent a ten-minute open field test during their light cycle, in which overall locomotor activity as well as activity in the periphery versus the center of a 75cm x 75cm box was measured over a ten-minute period. *Social Interaction:* Rats next underwent social interaction testing during the light cycle. In this test, they were placed into the same arena used for open field testing, now containing a novel younger male animal, and allowed to explore for ten minutes. Latency to interact, direction of interaction (test animal with experimental animal or vice versa), and total time interacting were recorded. *Elevated Plus Maze:* An elevated plus maze test performed during the dark cycle was used to model anxiety-

like behavior in the rats by measuring the time spent in open arms vs. the time spent in the closed arms (Pellow et al., 1985) (n=8-14 per group). The specifications for the San Diego Instruments elevated plus maze used were as follows: 43 ½" long, 4" wide (arm width), 19 ½" high (open arms), and 31 ½" high (closed arms). During testing, animals were able to freely move from open to closed arms for 5 min. *Forced Swim Test:* This test, originally designed to measure antidepressant efficacy, can be used to measure depressive-like behavior and coping strategies (Porsolt et al., 1977). During the light cycle, rats were placed in a clear acrylic beaker (40 cm high X 18 cm diameter) filled with room temperature water. Struggling, latency to float, and floating were analyzed. Floating was defined as the animal's limbs remaining motionless for at least two seconds, and struggling was defined as the animal's limbs in motion and its head above the surface. Immediately after the end of the 10 min test, rats were removed from the beaker and tail blood was collected by tail snip of less than two mm of the tail within two minutes and without anesthesia for corticosterone analysis, described below.

8.2.11 Lesion Volume Assessment One day after the end of behavioral testing (Surgical cohort 1, PND84; Surgical cohort 2, PND100), animals in the surgical cohorts were euthanized with Euthasol® perfused for two minutes with saline and ten minutes with 4% paraformaldehyde for ten minutes. Brains were removed and post-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and sectioned at 40 µm on a cryostat. In addition, adrenals and testes were collected and weighed. To assess lesion damage, sections (n=12 per animal, ssf=1/12) from sham (Chow, n=6; Fructose, n=6) and MCAO animals (Chow, n=9, Fructose, n=9) were stained with 0.1% cresyl violet and coverslipped with Permount (Fisher Scientific) before stereological assessment. The Cavalieri method was used to estimate mean hemispheric and lesion volumes of both the ipsilateral (right) hemisphere as well as volume of the contralateral (left) hemisphere, considering only cystic spaces as lesions for analysis. The right and left hemispheric volumes of sham animals were calculated by the same method. All stereological assessment was performed by a blinded research assistant using the Cavalieri probe from Stereoinvestigator (MBF

Bioscience, Williston, VT) on the same Nikon Eclipse 90i microscope (Melville, NY) described above.

8.2.12 Statistical Analysis: All statistics and graphing were performed using either GraphPad Prism Version 6.0 or RStudio Version 0.98.1049. Data were assessed for normality using the D'Agostino and Pearson normality test. Student's t-tests, Kolmogorov-Smirnoff tests, Analysis of Variance (ANOVA) type II tests, and Pearson's correlations were performed with $\alpha=0.05$. T-tests are two-tailed unless otherwise noted; a Welch's correction was used with parametric data with non-equal variances. After ANOVA, secondary analyses for planned contrasts or Holm-Sidak post-hoc tests were performed when appropriate and are noted in the text.

8.3 Results:

8.3.1 Fructose alters basal metabolism and decreases weight loss after surgery. Fructose-fed rats in the Vascular cohort weighed more by nine weeks on the diet than chow-fed rats ($t_{34}=2.168$, $p=0.0373$, **Figure 8.2a**). Caloric efficiency was calculated as mg of weight gained per kCal consumed averaged over a week for the first six weeks on each diet. Fructose-fed rats had a higher caloric efficiency than chow fed rats (**Figure 8.2b**, $F_{1,21}=6.433$; $p=0.0192$), as previously found in Chapter Five. Fasting blood glucose was also assessed during this period, and fructose-fed rats similarly had elevated fasting blood glucose (**Figure 8.2c,** $F_{1,20}=14.46$; $p=0.0011$). In the surgical cohorts, rats were matched by weight so that sham and MCAO groups as well as both diet groups had equal average weights prior to surgery. After 72 hours post-surgery, both surgery (**Figure 8.2d**, $F_{1,38}=126.5$; $p<0.0001$) and diet ($F_{1,38}=10.40$; $p=0.0026$) significantly affected weight such that MCAO animals lost weight, and a Holm-Sidak post-hoc test showed that fructose-fed rats lost less weight after MCAO surgery ($p=0.0099$). Terminal weights in the surgical cohorts were not altered by diet at either time point (Cohort 1:

$F_{1,19}$=0.4171; p=0.5261; Cohort 2: $F_{1,18}$=1.048; p=0.3196), but MCAO reduced terminal weight of both chow-fed and fructose-fed rats in Cohort 1 ($F_{1,19}$=9.052; p=0.0072, data not shown).

8.3.2 Fructose increases hippocampal gene expression of Vegfa. Hippocampal expression of vascular endothelial growth factor-a (**Figure 8.3a;** *Vegfa*) was significantly increased at nine weeks in fructose-fed ($t_{8.685}$=3.272, p=0.0101).

8.3.3 Fructose increases vascular density in CA1 of the hippocampus. Vascular length was assessed using the Stereoinvestigator Spaceballs probe (MBF Biosciences, Williston, VT), with rat endothelial cell antigen (RECA) as a marker of endothelial cells. In this analysis, vascular length was significantly increased in the CA1 of fructose-fed rats as determined by a one-tailed Student's t-test (**Figure 8.3b,** t_{10}=2.177, p=0.0273), but vascular length did not differ between diet groups in the hippocampus as a whole (t_{10}=0.4531, p=0.3301) or in any other subregion (DG: t_{10}=0.1397, p=0.4458; CA2: t_{10}=0.1167, p=0.4547: CA3: t_{10}=0.7580, p=0.2330; data not shown). Representative images of the CA1 from chow and fructose-fed animals are shown in **Figure 8.3c** and **8.3d.**

Vascular length was also assessed in the amygdala. Amygdalar vascular length was unchanged between chow- and fructose-fed animals (t_4=0.6499, p=0.5454; **Figure 8.4a**). Representative images of the amygdala from chow and fructose-fed animals are shown in **Figure 8.4b** and **8.4c.**

8.3.4 Fructose does not alter blood brain barrier permeability. For BBB permeability, the percentage of the brain immunoreactive for IgG was calculated using the Gray Level Index to quantify mean optical density for each brain (ImageJ, NIH). However, fructose-fed and chow-fed brains did not differ in mean optical density when stained for IgG (**Figure 8.5a**, t_{16}=0.7816, p=0.4459), indicating no difference in blood brain barrier permeability. Representative images of IgG staining in chow and fructose-fed animals are shown in **Figure 8.5b** and **8.5c.**

8.3.5 Fructose and acute stress increase corticosterone irrespective of surgery. A three-way repeated-measures ANOVA type II was used to compare corticosterone levels in chow- and

fructose-fed sham and MCAO animals at baseline or after a ten-minute forced swim. Both fructose (**Figure 8.6**, $F_{1,40}=4.531$, $p=0.0395$) and the forced swim test ($F_{1,40}=196.2$, $p<0.0001$) increased serum corticosterone levels as main effects without an effect of surgery ($F_{1,40}=0.9660$, $p=0.3316$) or any interactions (all $p>0.30$).

8.3.6 Fructose does not exacerbate MCAO-induced impairments in neurologic behavior. In the open field, repeated measures ANOVA indicated only an effect of repeat testing (pre vs. post-surgery; $F_{1,42}=20.75$; $p<0.0001$) that reduced distanced traveled, but neither diet ($F_{1,42}=0.4936$; $p=0.4861$), surgery ($F_{1,42}=1.411$; $p=0.2416$), nor any interactions (all $p>0.45$) altered locomotor activity. Given this effect of repeat testing, pre- and post-tests were analyzed separately. In the post-test for open-field, surgery tended to reduced distance traveled among the MCAO cohorts (**Figure 8.7a**, $F_{1,42}=3.801$, $p=0.0579$) without any effect of diet ($F_{1,42}=0.8657$, $p=0.3575$) or an interaction between diet and surgery ($F_{1,42}=0.0009$, $p=0.9763$). Pre-surgery, there was no effect of surgery, diet, or an interaction (**Figure 8.8a**, all $p>0.85$) on distance traveled.

In the sticky dot test for removal of a dot from the contralateral (left) paw, results showed a significant interaction between surgery and repeat testing (pre or post; $F_{1,42}=9.899$, $p=0.0030$), but no independent main effects of diet, surgery (all $p>0.15$). Given the interaction with repeat testing, pre- and post-tests were analyzed separately. In the post-test, surgery increased removal time in the MCAO cohorts (**Figure 8.7b**, $F_{1,42}=4.441$, $p=0.0411$) without any effect of diet ($F_{1,42}=0.0308$, $p=0.8618$) or an interaction between diet and surgery (**Figure 8.8b**, $F_{1,42}=0.5088$, $p=0.4796$). Pre-surgery, surgery reduced removal time in the animals that would receive MCAO ($F_{1,42}=8.061$, $p=0.0069$), while fructose tended to increase removal time ($F_{1,42}=3.295$, $p=0.0766$).

In the rotorod test, results indicated a significant main effect of the MCAO surgery ($F_{1,42}=5.130$, $p=0.0287$) as well as a significant effect of repeat testing ($F_{1,42}=10.31$, $p=0.0025$), but no effect of diet ($F_{1,42}=0.7032$, $p=0.4065$) or any interactions (all $p>0.25$). Given this effect of repeat testing, pre- and post-tests were analyzed separately. In the post-test, surgery altered

latency to fall from the rotorod (**Figure 8.7c**, $F_{1,42}$=4.198, p=0.0468) without an effect of diet ($F_{1,42}$=0.7767, p=0.3832). In the pre-test, diet (**Figure 8.8c**, $F_{1,42}$=0.4003, p=0.5304) had no effect on rotorod behavior, though there was a trend for intended surgery ($F_{1,42}$=3.555, p=0.0663) to alter rotorod behavior. In the grip strength test, a trend existed for diet to reduce grip strength in the three-way ANOVA ($F_{1,42}$=2.979, p=0.0917), but neither surgery ($F_{1,42}$=1.174 p=0.2848), repeat testing ($F_{1,42}$=1.828, p=0.1836), nor any interactions (all p>0.15) affected performance otherwise (data not shown).

8.3.7 Fructose promotes anxiety-like behavior in both sham and MCAO-affected animals Affective-type behaviors, including both anxiety-like and depressive-like behaviors, were examined two weeks post-surgery over a period of five days with the following tests: light-cycle open field to assess anxiety-like behavior, social interaction to assess social withdrawal and anhedonia, elevated plus maze to assess anxiety-like behavior, and the forced swim test to assess depressive-like behavior.

Grooming behavior is one index of anxiety-like behavior that increases with stress exposure and can be analyzed in the open field (Kalueff & Tuohimaa, 2004). In this study, fructose-fed animals spent significantly less time grooming (**Figure 8.9a**, $F_{1,40}$=5.666, p=0.0222) without an effect of surgery ($F_{1,40}$=0.0430, p=0.8367) or an interaction between diet and surgery ($F_{1,40}$=0.0372, p=0.8479). Indicating recovery from surgery at this time point and consistent with findings in Chapter Five, results indicated a trend for fructose-fed rats to travel farther in the open field during the light cycle (**Figure 8.9b**, $F_{1,40}$=3.761, p=0.0595), but no effect of either surgery ($F_{1,40}$=1.486, p=0.2300) or an interaction between surgery and diet ($F_{1,40}$=0.5036, p=0.4820).

The social interaction test is a test of both anxiety-like behavior as well as anhedonia (File & Hyde, 1978). Data showed that fructose-fed animals had significantly increased latency to social contact (**Figure 8.9c,** $F_{1,38}$=6.603, p=0.0142), but neither surgery alone ($F_{1,38}$=0.1778, p=0.6758) nor in interaction with diet ($F_{1,38}$=0.1778, p=0.6758) altered latency to social contact. Total duration of active social contact tended to decrease among fructose-fed groups ($F_{1,38}$=2.905,

p=0.0965; data not shown) but again surgery did not affect social contact behavior either alone or in interaction with diet (both p>0.80).

In the elevated plus maze, increased time or entries into the closed arms of the maze indicates anxiety-like behavior (Pellow et al., 1985). Here, diet, surgery, nor an interaction thereof affected closed arm or open arm entries (all p>0.25; data not shown). However, the MCAO surgery reduce affect time spent in the very center of the elevated plus maze (**Figure 8.9d;** $F_{1,42}$=6.889, p=0.0120), and Holm-Sidak post-hoc testing showed that fructose-fed MCAO animals spent significantly less time in the center of the maze than fructose-fed sham animals (p=0.0302). There was no main effect of diet or an interaction of diet and surgery on time spent in the center (both p>0.10).

8.3.8 Greater lesion volume correlates with an active coping strategy in the forced swim test As previously noted, the forced swim was assessed during behavioral testing two weeks post-injury test to assess depressive-like behavior. Reduced struggling behavior, reduced latency to immobility, and increased immobility are indicators of behavioral despair and depressive-like behaviors. These behaviors were analyzed by type II two-way ANOVAs and Holm-Sidak post-hoc tests (factors of diet and surgery) as well as Pearson correlations to lesion-free hemisphere volume. In the two-way ANOVA, no significant effects of diet, surgery, or an interaction between diet and surgery observed in any behavior (all p>0.10, data not shown). However, greater lesion-free hemisphere volume was inversely correlated with latency to become immobile (**Figure 8.10a,** r=-0.4553, p=0.0501) and positively correlated with time spent immobile (**Figure 8.10b,** r=0.5689, p=0.0110).

8.3.9 Fructose does not change lesion volume after MCAO Lesion and hemispheric volumes were assessed by the Cavalieri method counting only cystic spaces as lesion, and the volume of the contralateral hemisphere minus the lesion was calculated. Two-way type II ANOVA of the lesion-free hemisphere volume indicated that MCAO surgery reduced lesion-free volume (**Figure 8.11a,** $F_{1,27}$=4.569, p=0.0418) but that the fructose diet had no effect on lesion

volume ($F_{1,27}$=0.492, p=0.4889) nor was there an interaction between fructose and surgery ($F_{1,27}$=2.708, p=0.1115). Direct analysis of lesion volume in MCAO animals indicated no difference between chow-fed and fructose-fed animals (**Figure 8.11b;** t_{17}=0.2383; p=0.8145). Significant variability was evident in lesion volume, and representative images from chow-fed and fructose-fed sham and MCAO animals are shown in **Figures 8.11c- h**. Percent occlusion and percent reperfusion were also analyzed between chow- and fructose-fed MCAO animals, and there was no effect of either diet on percent occlusion (t_{19}=.6975, p=0.4939) or reperfusion (t_{19}=0.7893, p=0.4397; data not shown), nor was there a relationship between percent reperfusion and lesion-free volume (r=-0.1506, p=0.5383) or percent occlusion and lesion-free volume (r=0.3676, p=0.1216; data not shown).

4. Discussion:

Depression is a common sequela of stroke (Whyte et al., 2004), but its etiological underpinnings remain poorly understood. Metabolic dysfunction can promote depressive symptoms (R. J. Anderson et al., 2001; Lamers et al., 2013), increase stroke risk (Reilly & Kelly, 2011), and increase risk for development of post-stroke depression (Tanislav et al., 2015). We hypothesized that a high-fructose diet initiated in adolescence would create a metabolic and cerebrovascular environment that would be vulnerable to the “second hit” of an ischemic event, exacerbating post-stroke outcomes. In light of this hypothesis, we demonstrated that periadolescent fructose-fed rats had increased hippocampal *Vegfa* gene expression and vascular length in region CA1 (**Figure 8.3**) without changes in blood-brain-barrier permeability (**Figure 8.5**). This diet also increased baseline corticosterone (**Figure 8.6**) and promoted anxiety-like behavior in both sham animals and after MCAO surgery (**Figure 8.9**). However, fructose did not alter neurologic behavior (**Figures 8.7 & 8.8)** or lesion volume (**Figure 8.11**) after ischemia. In addition, MCAO did not influence affective-behavior (**Figure 8.9**). Collectively, the data

presented herein indicate that a high-fructose diet consumed during adolescence alters cerebrovasculature, basal HPA axis output, and affective behavior in male rats, but it does not exacerbate outcomes after MCAO.

The fructose-induced increase in CA1 vascular length (**Figure 8.3**), though contrary to the original hypothesis, is consistent with the hyperglycemia literature. The Goto-Kakizaki diabetic rat, a model of non-obese type 2 diabetes, shows increased neovascularization, which can be protective during ischemic events but at greater risk of hemorrhagic transformation (W. Li et al., 2010). Both animal and human studies have resulted in the hypothesis that hyperglycemia increases arterial resistance to increase vascular tone and reduce cerebral blood flow (Ergul, Li, Elgebaly, Bruno, & Fagan, 2009). During acute stress-induced hyperglycemia, such as in stroke, this effect is deleterious and enlarges infarct size and edema. However, chronic moderate hyperglycemia allows for neovascularization in response to increased arterial resistance that can promote neuronal preconditioning and reduce infarct size. Nonetheless, the risk for edema and hemorrhagic transformation remain (Ergul, Kelly-Cobbs, Abdalla, & Fagan, 2012; Ergul et al., 2009).

In the present study, the apparent regional specificity of increased vascular length is particularly interesting. Neither the hippocampus examined in entirety nor the amygdala (**Figure 8.4**) showed increases in vascular length. While this may in part be explained by reduced power to observe significant differences in the amygdala, given the smaller sample size used, vascular regional specificity may also be linked to dynamic interactions between glycemic associated angiogenic factors and hypercorticosteronemia, as observed in the fructose-fed animals (**Figure 8.6).** Increased hippocampal cell proliferation in the context of diabetes has been proposed as a compensatory mechanism to the neuronal suffering induced by excessive glucocorticoid exposure (Beauquis et al., 2010). The CA1 region of the hippocampus may be especially vulnerable to glucocorticoid-mediated effects. For example, early-life stress reduces CA1 long-term potentiation and promotes depressive-like behavior (Cui et al., 2006). Other studies have shown

that adulthood stress can promote depressive-like behavior (Hollis, Wang, Dietz, Gunjan, & Kabbaj, 2010) and adulthood stress can reduce hippocampal vasculature, an effect exacerbated by prenatal glucocorticoid exposure (Neigh, Owens, Taylor, & Nemeroff, 2010). In the Goto-Kakizaki rat, a model of spontaneous Type 2 diabetes, elevated circulating glucocorticoids and glucose are linked to reductions in expression of the glucocorticoid receptor in the CA1 (Beauquis et al., 2010). In the present study, the fructose-fed rats were also simultaneously confronted with chronic hyperglycemia (**Figure 8.2**) and elevations in basal plasma glucocorticoids (**Figure 8.6**). Chronic exposure to elevated glucocorticoids in the context of hyperglycemia may have enhanced a potentially adaptive physiological response to induce vascularization in the CA1.

The increase in hippocampal *Vegfa* expression (**Figure 8.3a**) observed in fructose-fed rats is also consistent with the increased CA1 vascularization. VEGF-A, one of five members of the vascular endothelial growth factor family, is a secreted mitogen specific to vascular endothelium that plays an important role in angiogenesis and maintenance of the blood-brain-barrier (Beck & Plate, 2009; Rosenstein, Mani, Silverman, & Krum, 1998), and may also affect neurogenesis (Jin et al., 2002). VEGF, widely expressed in the normal brain in endothelial cells, neuron, and astrocytes, is upregulated in response to both physiologic (Ogunshola et al., 2000) and pathologic (Beck & Plate, 2009) hypoxia. Angiogenesis in response to this hypoxia is mediated by the binding of VEGF to its receptors, primarily to specific VEGF receptors that dimerize and result in subsequent signal transduction, but also to the neuropilin co-receptors (Beck & Plate, 2009). VEGF also mediates increased permeability of the blood-brain barrier (BBB)(Mayhan, 1999), which stands in contrast to the lack of difference in BBB permeability in the present study (**Figure 8.5**). However, the present study assessed VEGF expression in a region-specific manner, and increased vascularization also appeared to be region specific (**Figure 8.4**). Regional analysis of BBB permeability may demonstrate region-specific changes in permeability that would be more consistent with the increased VEGF expression. In addition,

examination of specific BBB proteins (i.e., claudins, occludins) could permit more nuanced understanding BBB changes in the context of the high-fructose diet. Together, however, consumption of this diet during periadolescence was consistent with indicators of vascular remodeling in the hippocampus and CA1 in particular.

The high-fructose diet consumed during adolescent development additionally promoted anxiety-like behavior in animals after both sham and MCAO surgeries (**Figure 8.9**). This finding is consistent with findings in Chapter Five, though notably in different behavioral tests. Here, fructose-fed animals displayed reduced grooming in the open field and increased latency to interact in the social interaction test. They also tended to travel farther in the open field, indicating no effect of either diet or surgery on long-term mobility (**Figure 8.9**). Intriguingly, the present study did not replicate prior results in the elevated plus maze or the forced swim test (see **Chapter Five**). However, both these tests showed some effects of surgery on behavior. In the elevated plus maze, MCAO surgery reduced time that fructose-fed animals spent in the open center of the maze (**Figure 8.9d**), potentially impairing our resolution of the differences in open and closed arm entries between groups. In the forced swim test, lesion size correlated with less time immobile and an increased latency to float (**Figure 10**), indicating an active coping strategy associated with more severe MCAO. Winters et al (Winter et al., 2005) found a similar hyperactive phenotype in the forced swim test in 129/Sv mice after right-sided MCAO. Decreased activity after MCAO has typically been seen only in conjunction with an additional stressor (O'Keefe et al., 2014).

Consumption of a high-fructose diet during adolescence did not alter either neurologic behavior (**Figures 8.7 & 8.8**) or lesion volume (**Figure 8.11**). Given that the stress-induced elevation in serum corticosterone did not differ between chow-fed and fructose-fed animals (**Figure 8.6**), it is possible that both groups experienced similar levels of intra-surgery glucocorticoids, and thus would not see the differential effects of glucocorticoid exposure on lesion volume that has been previously established (Craft & DeVries, 2006). Given that there

was no difference in blood-brain-barrier permeability (**Figure 8.5**) and that the increase in vascularization observed could be a result of adaptive neovascularization in response to hyperglycemia (Ergul et al., 2009), the fructose-fed animals were not at an additional risk from the MCAO due to cerebrovascular changes, though they were not spared damage, either. It is additionally possible that pro-inflammatory mechanisms could be altered between the fructose-fed and chow-fed groups either before or after surgery, though these were not examined in the present study. Ischemia and ischemia-associated depressive-like behavior are associated with a pro-inflammatory state (Craft & DeVries, 2006), as are hyperglycemia (Wellen & Hotamisligil, 2005) and adiposity (Lyon, Law, & Hsueh, 2003), both seen in the high-fructose diet model used in this study (**Figure 8.2**, **Chapter Five, Chapter 7**). Future studies may explore the relationship between behavioral outcomes and an inflammatory profile.

Both the sex and age of the animals used in this study could be important contributor to the observed results. Females are at a twofold risk of depression in the general population (Kessler et al., 2003) and may be at increased risk of post-stroke depression (Poynter et al., 2009). However, this increased risk for post-stroke depression is not consistently reported (Poynter et al., 2009). The 2:1 female predominance in depression is tempered in several comorbid disease states, which may be related to interactions between the pro-inflammatory state somatic disease and sex hormones (Nemeth, Harrell, Beck, & Neigh, 2013). Data from animal studies is consistent with this hypothesis; after microembolism, a different model of cerebral ischemia, male rats appear to be more vulnerable to depressive- and anxiety-like behavior than female rats, despite an equal microglial response (Nemeth, Reddy, Bekhbat, Bailey, & Neigh, 2014). Female sex steroids may have protective effects to modify the functional consequences of this inflammation (Nemeth et al., 2014; Pyter et al., 2013). As for age, the animals used in the present study were all tested in young adulthood, while on the high-fructose diet that had been initiated just after weaning. These time points were selected given the known effects of the periadolescent diet during young adulthood and the growing epidemic of metabolic dysfunction in youth (Ogden

et al., 2014). While stroke and post-stroke depression occur in young populations (Tanislav et al., 2015), their prevalence increases in older populations (Whyte et al., 2004). Increased length on the high-fructose diet may exacerbate the metabolic phenotype, resulting in increased vulnerability. Future studies on the effects of a high-fructose diet and MCAO in both sexes as well as in older animals will be necessary to determine the impact of age and sex this model.

Stroke laterality may have influenced the findings in the present study. In the human literature, the traditional hypothesis has held that left hemisphere strokes are more frequently associated with vascular depression (Bhogal, Teasell, Foley, & Speechley, 2004). However, recent studies have called this hypothesis into question, noting that the direction of association can change depending on the location and timing of patient sampling (Bhogal et al., 2004) and a recent meta-analysis of 43 clinical studies concluded that there was a significant association between vascular depression and right hemisphere lesions when assessed within six months of the event (N. Wei et al., 2015). Others have proposed that we discard lesion location hypothesis entirely and shift our focus toward the effect of vascular burden and the accumulation of micro- and macrovascular lesions on vascular depression (Santos et al., 2009). Given that the role of laterality on affective-behavior is even less well-established in the rodent literature, there is insufficient evidence to determine whether lesion location played a role in the current findings.

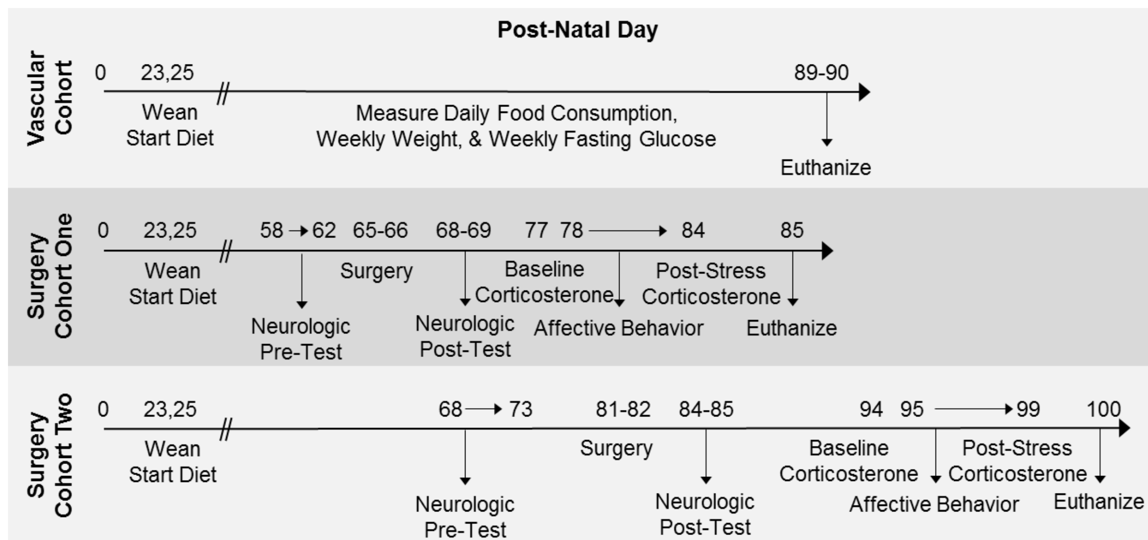
In conclusion, a high-fructose diet consumed during adolescent development increases vascularization of region CA1 of the hippocampus, elevates HPA axis output at baseline, and induces anxiety-like behavior in adulthood after sham surgery or after transient ischemia induced by MCAO surgery in male rats. In terms of neurologic behavior and lesion size after MCAO, adaptive responses to the metabolic influence of the high-fructose diet may result in equivalent outcomes in spite of the diet's ability to act as a chronic stressor. Future studies will be necessary to assess additional factors that affect human well-being, such as the effect of fructose on a pro-inflammatory profile and the effect of treatments to counteract the metabolic, hormonal, and behavioral effects of fructose.

Table 8.1.

Cohort	Diet	Surgery	Experimental Use	N
Metabolic & Vascular Cohort	Chow	Not applicable	Weight, Fasting Blood Glucose, Corticosterone ELISA, PCR (*Vegf*)	8
	Fructose			10
	Chow		Weight, Fasting Blood Glucose, Immunohistochemistry (IgG, n=8; RECA, n=6)	8
	Fructose		Weight, Fasting Blood Glucose, Immunohistochemistry (IgG, n=10, RECA, n=6)	10
Surgery Cohort 1 (PND 65-66)	Chow	Sham	Open Field Dark Cycle (OF-D), Sticky Dot, Grip Strength, Rotorod, Open Field Light Cycle (OF-L), Social Interaction (SI), Elevated Plus Maze (EPM), Forced Swim Test (FST), Corticosterone ELISA, Immunohistochemistry (Nissl; n per group: Chow-Sham=5; Chow-MCAO=6; Fructose-Sham=5; Fructose-MCAO=4)	6
		MCAO		6
	Fructose	Sham		6
		MCAO		5*
Surgery Cohort 2 (PND 81-82)	Chow	Sham	OF-D, Sticky Dot, Grip Strength, Rotorod, Open Field OF-L, SI, EPM, FST, Corticosterone ELISA, Immunohistochemistry (Nissl; n per group: Chow-Sham=1; Chow-MCAO=3; Fructose-Sham=1; Fructose-MCAO=5)	6
		MCAO		5*
	Fructose	Sham		6
		MCAO		5*

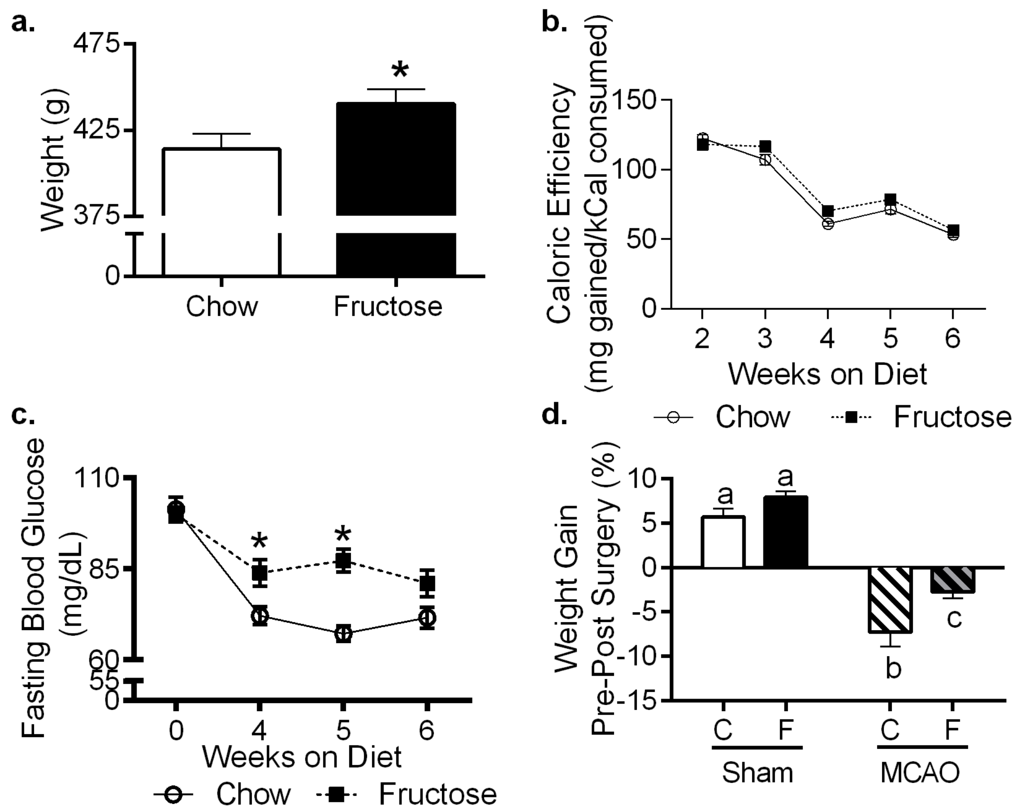
Cohort Distribution and Use This lists the number and use of animals in Chapter 8. For a full list of animals used in the high-fructose diet experiments, see Chapter 5. **Indicates one animal died during surgery or in the post-surgical period. Original n=6.*

Figure 8.1



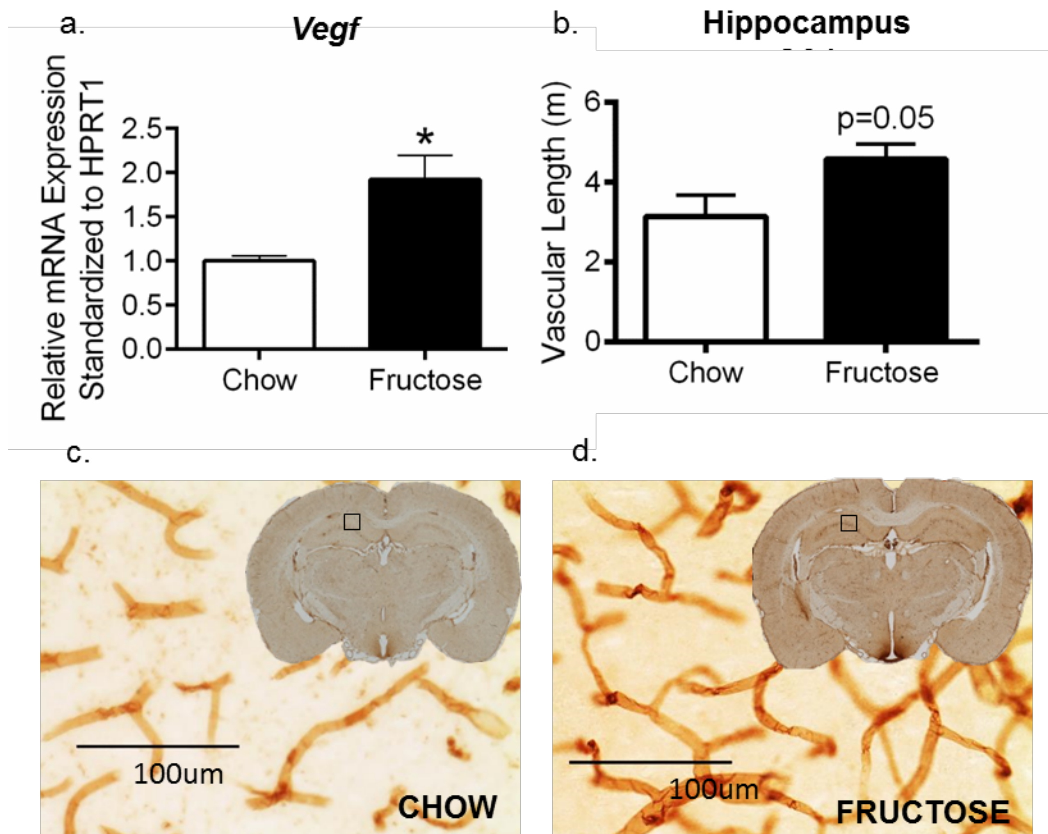
Dietary, surgical, and behavioral timelines for cohorts used in this study. The experimental timelines for the three cohorts used in this study are displayed. Neurologic behavioral assessment included the following tests: open field behavior during the animals' dark cycle, grip strength, sticky dot, and rotorod. Affective behavioral assessment included the following tests: open field behavior during the animals' light cycle, elevated plus maze, social interaction, and forced swim. Post-natal days are not shown to scale in order to enhance readability.

Figure 8.2.



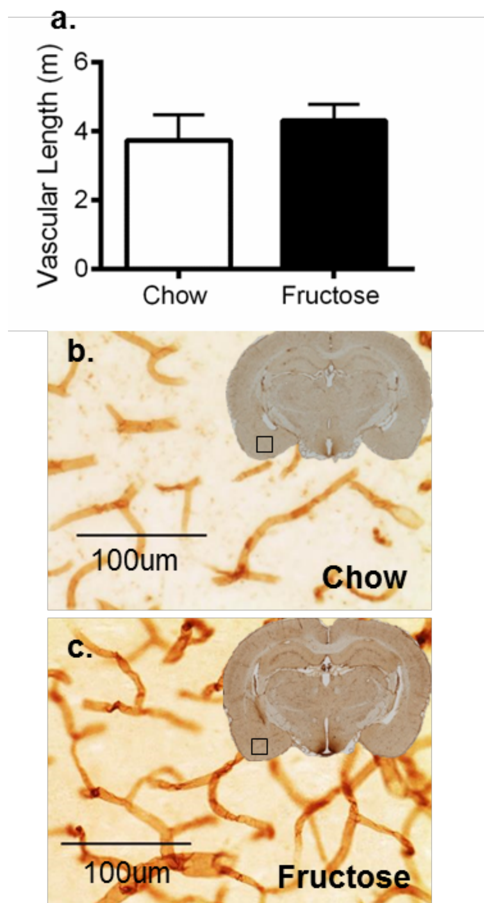
Fructose alters basal metabolism and decreases weight loss after surgery a. Fructose-fed rats weighed more than chow-fed rats after nine weeks on the diet Asterisk indicates significant difference versus chow-control ($p<0.05$). b,c. Among the rats in the surgical cohorts, fructose-fed rats had a higher caloric efficiency and fasting blood glucose than chow fed rats prior to surgery. Asterisks indicate significant post-hoc differences versus chow blood glucose levels at each time-point ($p<0.05$). d. Both diet and surgery type significantly affected weight 72 hours after surgery, such that MCAO animals lost more weight than sham animals, and fructose-fed rats lost less weight after MCAO surgery than chow-fed animals. Letters indicate significant differences in post hoc testing ($p<0.05$). Data shown are mean ± SEM.

Figure 8.3



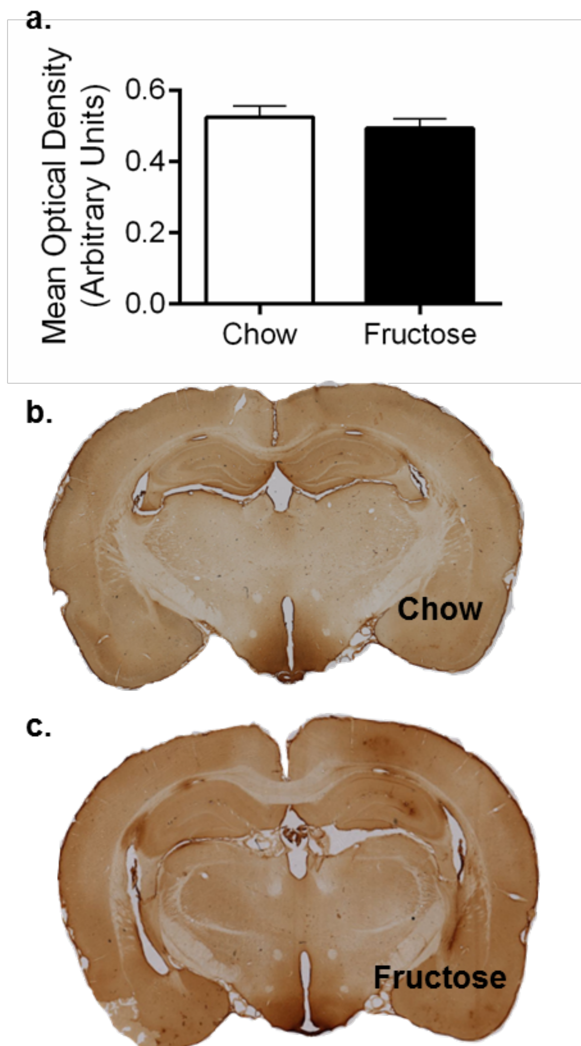
Fructose promotes hippocampal vascularization a. Hippocampal gene expression of vascular endothelial growth factor-a (*Vegfa*) was significantly increased at nine weeks in fructose-fed rats. b. Fructose-fed rats had increased CA1 vascular length relative to chow-fed rats as determined by stereological analysis of rat endothelial cell antigen (RECA) immunostaining. Representative images of Chow-fed (c) and Fructose-fed (d) RECA staining at 2x (hippocampus) and 40x (CA1). Data shown are mean ± SEM; asterisks indicate an effect at $p < 0.05$.

Figure 8.4



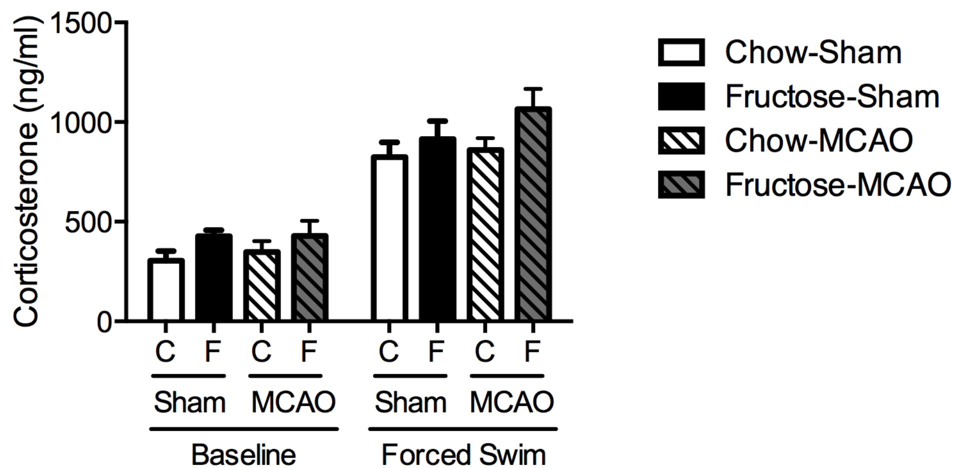
Fructose does not alter amygdalar vascularization a. Fructose-fed did not differ in amygdalar vascular length relative to chow-fed rats as determined by stereological analysis of rat endothelial cell antigen (RECA) immunostaining. Representative images of Chow-fed (c) and Fructose-fed (d) RECA staining at 2x (hippocampus) and 40x (CA1). Data shown are mean ± SEM; asterisks indicate an effect at $p < 0.05$.

Figure 8.5.



Fructose does not alter blood brain barrier permeability a. Fructose-fed and chow-fed brains did not differ in mean optical density when stained for IgG, a marker of blood-brain-barrier permeability. Representative Chow-fed (b) and Fructose-fed (c) sections stained for IgG.

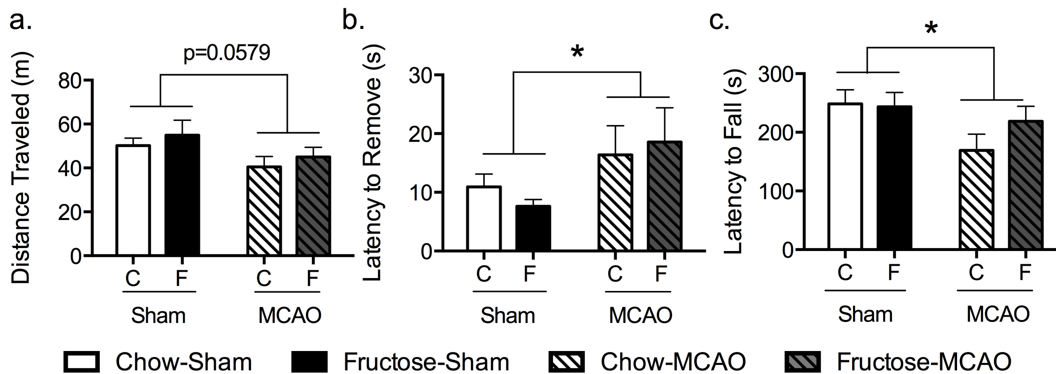
Figure 8.6.



Fructose and acute stress increase corticosterone irrespective of surgery A three-way repeated-measures ANOVA type II was used to compare corticosterone levels in chow- and fructose-fed sham and MCAO animals at baseline or after a ten-minute forced swim. Both the fructose diet and a ten-minute forced swim test increased serum corticosterone levels as main effects without an effect of surgery. Data shown are mean ± SEM; asterisks indicate an effect at $p < 0.05$.

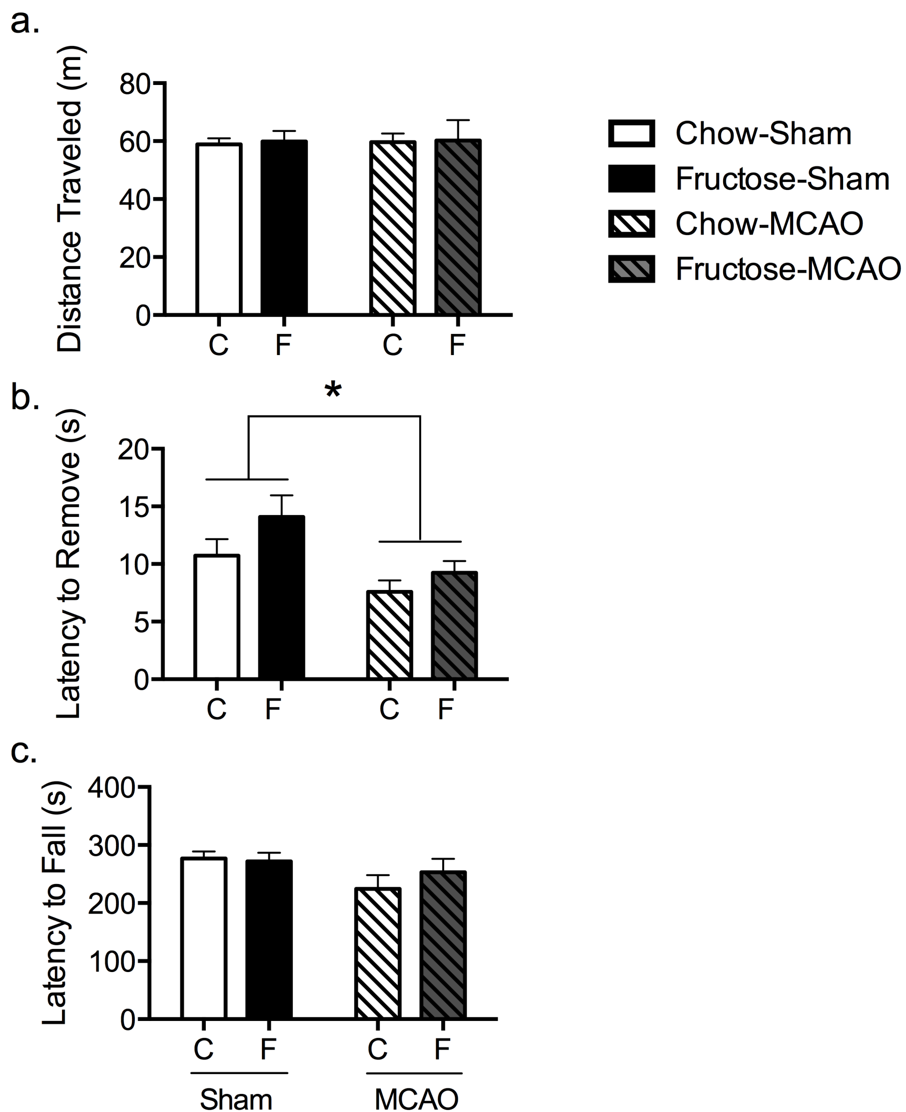
C=Chow, F=Fructose

Figure 8.7.



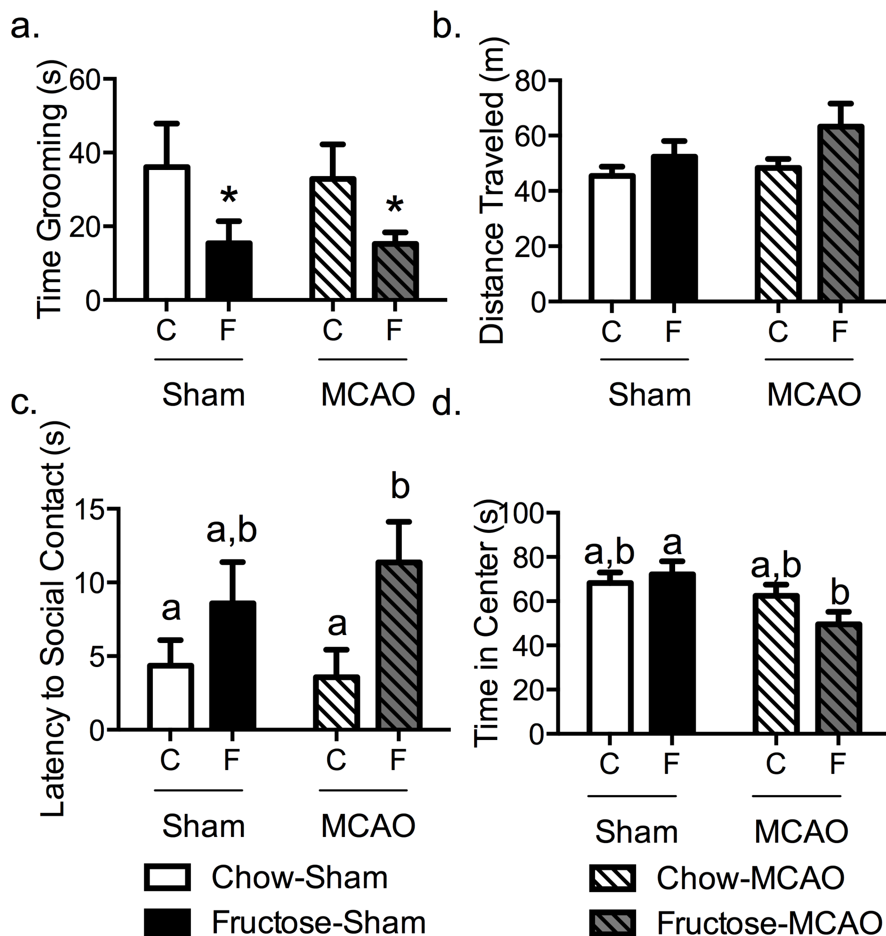
Fructose does not exacerbate MCAO-induced impairments in neurologic behavior a. Post-surgery, MCAO tended to reduce locomotor activity in the open field during the dark cycle. b. MCAO surgery increased removal time of a sticky dot from the left paw. c. MCAO surgery reduced latency to fall from the rotorod. No effects of diet or interactions of diet with surgery were observed. Data shown are mean ± SEM; asterisks indicate a main effect at $p < 0.05$. C=Chow, F=Fructose

Figure 8.8



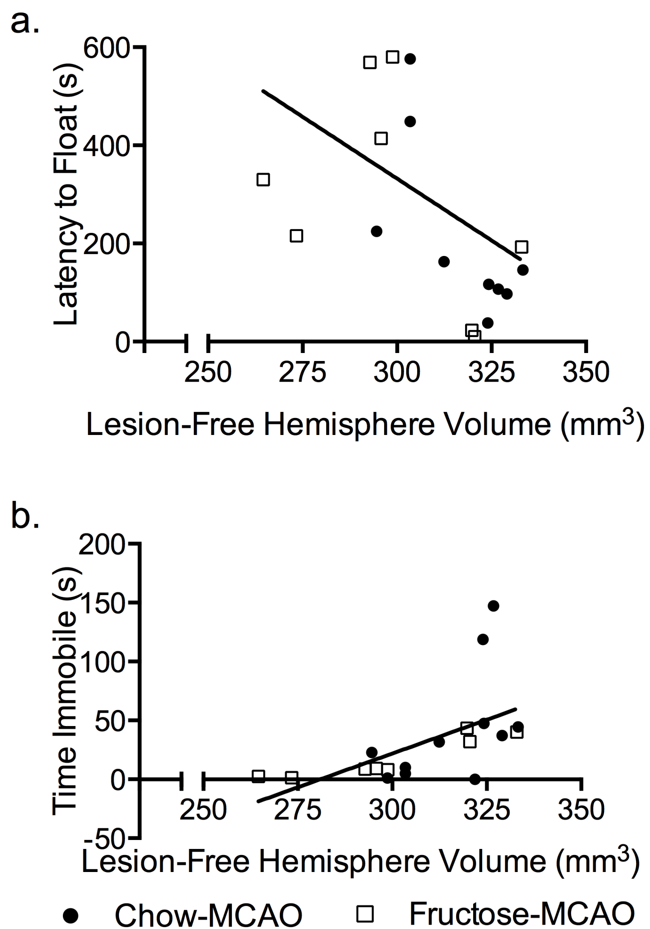
Baseline parameters in neurologic behavior a. Pre-surgery, there was no effect of diet, surgery group, or an interaction on distance traveled in the dark cycle open field test. b. Placement in the group intended for MCAO surgery reduced time to remove the sticky dot from the left paw, with no effect of diet or an interaction between surgery group and diet. c. Placement in the MCAO surgery group tended to reduce latency to fall in the rotorod group without an effect of diet or an interaction prior to surgery. Data shown are mean ± SEM; asterisks indicate a main effect at $p < 0.05$. C=Chow, F=Fructose

Figure 8.9.



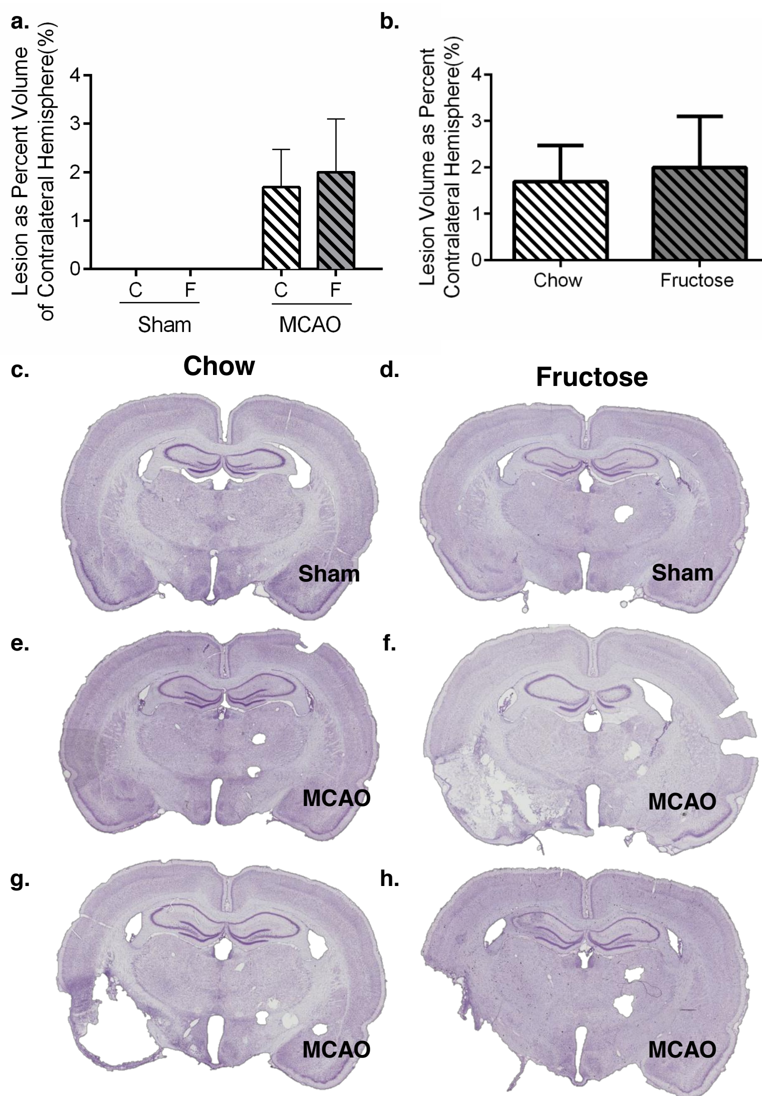
Fructose promotes anxiety-like behavior in both sham and MCAO-affected animals Fructose-fed animals spent significantly less time grooming (a) but tended to travel farther (b) in the light-cycle open field. c. Fructose-fed animals had significantly increased latency to social contact. Surgery did not affect these behaviors. d. MCAO surgery reduced time in the center of the elevated plus maze, and fructose-fed MCAO animals spent less time in the center of the maze than fructose-fed sham animals. Data shown are mean ± SEM; asterisks indicate main effects and letters indicate post-hoc effects with $p < 0.05$.

Figure 8.10.



Greater lesion volume correlates with an active coping strategy in the forced swim test Greater lesion-free hemisphere volume inversely correlated with latency to float in the forced swim test (a; $p=0.0501$) and positively correlated with immobility (b; $p<0.05$).

Figure 8.11.



Fructose does not alter lesion volume after MCAO. a. MCAO surgery increased lesion volume as a percent of the contralateral hemisphere. b. Chow- and fructose-fed animals did not differ in lesion volume after MCAO. c-h. Significant variability was evident in lesion volume, and representative images from chow-fed and fructose-fed sham and MCAO animals are shown. Data are shown as mean ± SEM.

GENERAL DISCUSSION

Chapter Nine

Energetic stress:
Expensive effects on body and brain

9.0 Abstract

This thesis has explored the energetic costs of multiple stressors, including psychosocial stress, inflammatory stimuli, hormonal fluctuations, dietary modulation, and physical injury (stroke). Each of these stressors causes different energetic effects, including shifts in glucose transporters, changes in weight and caloric efficiency, and alterations in the use and storage of energy. In turn, changes in energy availability have shifted the stress response. Psychosocial stress-induced changes in glucose transporters altered the responsiveness of these same transporters to an inflammatory stressor in a sex-dependent manner. Hormonally-influenced fluctuations in glucose transporters likely affected the extent to which stress shifted expression in females. High-fructose diet-induced metabolic dysfunction was most pronounced with the diet was introduced during an energetically demanding period of development, adolescence. This dysfunction coincided with altered affective behavior and changes in HPA axis output as well as extensive remodeling of the hypothalamic transcriptome, notably related to gene expression in POMC processing, complement pathways, and dopaminergic signaling. These studies highlight the profound relationship between metabolism and the stress response. The fructose-induced shift in energy availability additionally altered regional cerebral vasculature, which may protect against further energetic insult in the form of stroke. Taken together, this thesis has demonstrated that the reciprocal relationship between energy availability and the stress response is far-reaching, affecting the immune system, the endocrine system, limbic circuitry, and cerebral vasculature.

9.1 Introduction

Consumption of free energy is fundamental to all life, from single-celled organisms to complex creatures such as *Homo sapiens*. Energy consumption is required for nearly all cellular processes, and when free energy is no longer available, a cell dies (Alberts, 2002). Thus, meeting nutrient demands is necessary for survival, growth, and reproduction (Illius, Tolkamp, & Yearsley, 2002). Brain tissue is particularly metabolically demanding, and humans support a surprisingly large brain with a greater-than-expected organ-specific metabolic rate (Aiello & Wheeler, 1995). This "expensive tissue" comes at a cost; during the evolution of *Homo erectus*, total daily energy expenditure increased up to 85% (Leonard & Robertson, 1997). The dietary strategy of cooking among early hominid foragers co-evolved with our large brains to provide food of high caloric density (Wrangham & Conklin-Brittain, 2003). Since the beginning of human existence, diet and feeding behavior have been essential to support brain development and survival.

In addition to meeting the basic energy requirements for growth and reproduction, organisms must also respond to and fend off challenges to survival, which is metabolically demanding. The stress response system has evolved for this purpose. The response to a stressor comes in two waves (Sapolsky, Romero, & Munck, 2000). First, the sympathetic nervous system increases secretion of catecholamines, the hypothalamus decreases release of gonadotropin releasing hormone, the pituitary decreases release of gonadotropins while increasing prolactin and growth hormone, and the pancreas increases secretion of glucagon. Pituitary arginine vasopressin and renal renin are also increased. In the second wave, hypothalamic and pituitary increases in CRF and ACTH stimulate adrenal release of glucocorticoids, and gonadal steroid secretion declines. The primary effects of the stress response are metabolic. Stored energy is catabolized and diverted to muscle with assistance from enhanced cardiovascular tone, immune function increases, reproductive physiology declines, and cerebral perfusion rates and cerebral glucose utilization increases (Sapolsky et al., 2000).

Acute stress is regulated by a negative feedback system mediated through the glucocorticoid receptor (Bourke et al., 2012). Glucocorticoids in the hippocampus, hypothalamus, and pituitary bind to the glucocorticoid receptor (GR), a transcription factor, to initiate homodimerization and nuclear translocation. Several co-chaperones influence GR sensitivity and mediate DNA binding. GR binding to specific glucocorticoid response elements regulates transcription of CRF and POMC related genes to decrease production of CRF and ACTH (Bourke et al., 2012). In the context of chronic stress, however, glucocorticoid resistance can develop, resulting in an impaired ability to shut down the stress response. In such conditions, stress-related pathologies develop, inducing neurobiological changes that manifest in behavioral symptoms, such as altered affect and cognition; immune dysfunction, such as reduced T cell function, and metabolic dysfunction, including insulin resistance and visceral obesity (reviewed in (A. H. Miller, Maletic, & Raison, 2009; Raison, Capuron, & Miller, 2006)).

Similarly, altered energy homeostasis, such as in the context of diabetes or obesity, can alter the stress response. As reviewed in Chapter One, altered energy homeostasis disrupts central glucocorticoid signaling, altering glucocorticoid diurnality and changing HPA axis sensitivity to stimuli (Bjorntorp & Rosmond, 2000; Rosmond et al., 2000). Changes in peripheral energy stores also affect peripheral glucocorticoid signaling, altering tissue-specific actions of 11βHSD1 (Rask et al., 2002b) and changes in 11βHSD1 expression can alter metabolic responses to psychosocial stress (Kotelevtsev et al., 1997). Excess energy stores also promote SNS hyperactivity (Esler et al., 2001), which can result in an exacerbated response to acute stress (Pasquali et al., 1996). Neuroendocrine and neuropeptide factors are likewise highly responsive to perturbations of energy homeostasis, and factors such as NPY and the melanocortins help regulate HPA axis activity (Ryan et al., 2014; Wahlestedt et al., 1987). Finally, excess energy storage in adipose tissue promotes chronic low-grade inflammation (Hotamisligil, 2008), and such inflammatory stimulation can result in long-term disruption of the HPA axis (A. H. Miller et al., 2009).

In this thesis, we have explored the reciprocal interactions between energy homeostasis and the stress response (**Figure 9.1**). In Part One (Chapters Two and Three), we examined the environmental and hormonal influences on markers of cerebral metabolism, namely, glucose transporters. In Chapter Two, we explored the effects of psychosocial and inflammatory stress on regional cerebral glucose transporter expression between sexes and across developmental ages. In Chapter Three, we established that ovarian steroid also modulate regional expression of these same transporters. In Part Two, we shifted the focus to examine how a dietary disruption to energy homeostasis – i.e., a high-fructose diet – would alter the stress response. In Chapter Five, we determined the effects of this diet on metabolic, hormonal, and behavioral outcomes, indicating that high-fructose diet initiated during adolescence promotes a negative metabolic phenotype as well as depressive- and anxiety-like behavior. We also demonstrated that a periadolescent high-fructose diet remodels the hypothalamic transcriptome, with a particular impact on POMC processing. In Chapters Six and Seven, we expanded on the analysis of hypothalamic transcriptome and demonstrate the effects of the diet on inflammatory and dopaminergic pathways. Finally, in Chapter Eight, we showed that the high-fructose diet remodels cerebral vasculature without affecting behavioral outcomes after cerebral ischemia. In this general discussion, we will now reflect on the findings in each chapter and explore areas for future research.

9.2. Stress effects on a marker of cerebral energy metabolism

9.2.1 Psychosocial and inflammatory stress change expression of cerebral glucose transporters in a region-, age-, and sex- specific manner Regulation of cerebral glucose utilization occurs primarily through glucose transporters (GLUT), which facilitate the movement of glucose across the blood-brain-barrier and into neurons and glia (Brown, 2000; B. S. McEwen & Reagan, 2004; Simpson et al., 2007). Environmental factors can alter GLUT expression

(Choeiri et al., 2005), and changes in GLUT alter metabolic function (Simpson et al., 2007). Considering GLUT as a potential metabolic marker, in Chapter Two we examined the effects of chronic mixed modality stress on regional expression of five GLUT isoforms in adolescent and adult male and female rats. Exposure to chronic stress differentially impacted GLUT isoform expression depending on the age and sex of the animal, and effects of stress were observed in the hypothalamus, hippocampus, and amygdala, but not the prefrontal cortex. In the short term (three days) after stress exposure males, GLUT tended to increase in expression after stress in a region- and isoform specific manner. Males were more susceptible than females to changes in GLUT expression, showing notable increases in hippocampal GLUT1 expression after stress in both adolescence and adulthood. In the analysis of the effects of adolescent stress on hippocampal GLUT expression in the long term, however, females were more susceptible, showing reduced hippocampal GLUT1 four and half weeks after adolescent stress.

In response to an inflammatory stressor (LPS), only males had altered GLUT expression, with increased GLUT1, GLUT5, and GLUT8 expression in control rats. A history of chronic adolescent stress four and a half weeks earlier, however, blunted this increase in all three isoforms, but again, had no effect in females. Inflammation is well known to affect glucose metabolism, potentially doubling glucose production rate (Michie, 1996), thus the increased GLUT expression in males was unsurprising. The lack of female response is consistent with previous work from our lab (Pyter et al., 2013). In this study, males had a unique neuroinflammatory response to LPS, while females had an attenuated response, potentially due to interactions between stress-induced changes in estradiol and microglia. The increase in cerebral GLUT in males may be necessary for their relatively higher neuroinflammatory response. Future studies could examine whether the stress-induced failure to sufficiently upregulate GLUT in response to an inflammatory stimuli in males is associated with worse outcomes or later behavioral phenotypes.

9.2.2 Ovarian hormones modulate expression of cerebral glucose transporters in a region- and isoform- specific manner We cannot fully rule out the effects of stress on female GLUT expression, however, because we know from Chapter Three that ovarian hormones influence regional GLUT isoform expression. In Chapter Two, while we assessed estrous cycle stage by vaginal lavage, we had insufficient power to determine whether any variability in GLUT expression was affected by cycle state. Thus, in Chapter Three, we formally tested the hypothesis that GLUT expression would fluctuate in a region-specific manner across the rat estrous cycle. We observed significant changes in GLUT1 expression in the prefrontal cortex as well as significant changes in GLUT1, GLUT3, and GLUT4 expression in the hippocampus across the estrous cycle. GLUT1 and GLUT3 expression was highest during pro-estrus while GLUT4 expression was highest during estrus, potentially reflecting changes in the progesterone to estradiol ratio, which surges during pro-estrus and begins to return to baseline again during estrus. Progesterone has been shown to increase GLUT1 and GLUT3 expression (X. Li et al., 2013), while estradiol may play a more important role regulating insulin sensitivity via the insulin sensitive GLUT4 (Barros et al., 2006).

Removal of ovarian hormones by ovariectomy was sufficient to significantly reduce hippocampal expression of GLUT1, GLUT3, and GLUT8, but not GLUT4, potentially reflecting the varying sensitivity of these transporters to progesterone and estradiol. These changes in GLUT may have clinical relevance given the growing interest in the protective uses of hormones replacement therapy (HRT) against cognitive decline (LeBlanc, Janowsky, Chan, & Nelson, 2001). This interest has sparked research into the potential mechanisms whereby ovarian hormones modulates neural function (B. McEwen, 2002) and potentially protect brain function (Yousuf, Sayeed, et al., 2014). Neuroimaging studies show that HRT, specifically estrogen replacement, increases cerebral glucose metabolism (Eberling, Reed, Coleman, & Jagust, 2000) and regional cerebral blood flow, which correlates with improved performance on neurocognitive testing (Resnick, Maki, Golski, Kraut, & Zonderman, 1998). While much future research is

necessary, this data indicating a modulatory role for ovarian hormones in GLUT expression is a first stepping stone toward future studies to better understand the precise nature of this relationship and its therapeutic implications.

9.2.3 Future directions in stress effects on GLUT The most fundamental issue to be addressed moving forward in this research is the relationship between GLUT gene expression and cerebral energy metabolism. We have been using GLUT gene expression as a marker of cerebral metabolism, given the brain's high metabolic demand for glucose (Sokoloff, 1960) and the essential role of GLUTs in regulating brain glucose utilization (Simpson et al., 2007). However, we have not examined either translation of this gene expression into functional GLUT proteins, nor have we examined changes in kinetic properties that would alter glucose uptake and cellular metabolism. Different GLUT have different kinetics, as the Michaelis constant (K_m) for GLUT2 (minimally expressed in cerebral tissue, but highly expressed in liver, pancreas, kidney and intestine) is 40 mmol/L, the K_m for GLUT1 is 20 mmol/L, and the K_m for GLUT4 is a mere 3 mmol/L (Brown, 2000). Thus, a substantially higher expression of GLUT4 would be necessary to affect glucose uptake, and similarly, changes in GLUT1 or GLUT2 might be expected to have a greater impact on cell metabolism. As seen in Chapter Three, GLUT4 was in low abundance in all regions studied, though it was relatively more abundant in the hypothalamus than in other regions. Moving forward, subsequent studies examining the effects of psychosocial and inflammatory stress or ovarian hormones on cerebral glucose metabolism will need to establish the functional relationship between GLUT expression, glucose uptake, and cellular metabolism. Experiments could include using radiolabeled 2-deoxyglucose and autoradiography to assess changes in regional uptake after psychosocial or inflammatory stress in males and females. To examine the effects that changes in GLUT expression and cerebral glucose uptake have on the stress response, we could silence or overexpress targeted specific GLUT isoforms (likely GLUT1) using viral vectors in a region-specific manner. To address whether the tissue-specific expression of GLUT matters (i.e., endothelial cell vs. astrocytic GLUT1) in the context of stress,

these same stress protocols could be used with mice expressing cre-recombinase under the control of a tissue-specific promoter could be crossed with mice expressing the floxed GLUT of interest. The present studies have provided a foundation indicating that research into the relationship between stress and GLUT-mediated changes in cerebral glucose utilization could yield fruitful results that to enable a better understanding of the relationship between the stress response and cerebral energy homeostasis.

9.3 Effects of shifting energy homeostasis on the stress response

9.3.1 Fructose as an energetic stressor Though glucose is the primary source of energy for the brain (Sokoloff, 1960), it is not the only source of metabolic fuel. Ketone bodies, derived from fatty acid metabolism, are used extensively during early development as well as during low carbohydrate conditions such as starvation, exercise, or diabetes (Laffel, 1999). Growing evidence also supports the astrocyte-neuron lactate shuttle hypothesis, in which astrocytes take up glucose and convert it to lactate through anaerobic metabolism. Lactate is then shuttled to the synaptic cleft for uptake by neuronal monocarboxylate transporters (Pellerin et al., 1998).

The relationship between fructose and cerebral metabolism remains unclear. However, fructose consumption has increased in the past several decades from a range of 3 to 28 g/day in 1977-1978 to a range of 32 to 75 g/day in 1999-2004 (Marriott 2009), leading to a growing need to understand still this relationship. Fructose crosses the blood-brain-barrier at a rate much lower than glucose (Oldendorf, 1971; Thurston et al., 1972). By-products of fructose metabolism, such as lactate, cross the blood brain barrier more readily (G. M. Knudsen et al., 1991). Fructose also promotes insulin resistance and elevates circulating triglycerides and fatty acids (Havel, 2005), a "perfect storm" for ketone body production. In addition, fructose can preserve ATP levels and partially preserve synaptic function in a hippocampal slice preparation, though this effect can be blocked by inhibition of either glucose transporters or monocarboxylate transporters (Izumi &

Zorumski, 2009), indicating that fructose may support neuronal metabolism in a manner similar to the astrocyte neuron lactate shuttle. Taken together, fructose appears to only partially support cerebral metabolism, potentially leading to a state of energetic stress.

Fructose's actions on peripheral tissues are better understood and can also mediate energetic stress. Initially, fructose fails to produce the same satiety signals as glucose, and may promote excess consumption (Teff et al., 2009). As a reducing sugar, fructose produces reactive oxygen species by covalently bonding to a protein or lipid without enzymatic control, a process known as glycation (Kaneto et al., 1996). Fructose consumption results in dyslipidemia, hypertension, insulin resistance, and hyperuricemia (Tappy & Le, 2010). Such peripheral metabolic disruption may then react to alter cerebral energy homeostasis.

In Chapters Five through Eight, we explored the effects of a 55% high fructose diet on metabolic, hormonal, behavioral, and molecular outcomes. The primary goal of using this diet was to elicit physiologic changes typically associated with fructose consumption in humans, including altered lipid storage and hyperglycemia (Havel, 2005; Tappy & Le, 2010), and not to mimic common human consumption. This diet is substantially higher than that the average fructose consumption reported in the United States, which is currently estimated at about 10% of total caloric intake for the population on average, and highest among adolescent consumers, with one fourth of adolescents consuming at least 15% of daily calories from fructose (Vos et al., 2008). However, these percentages do not reflect the increase in mass of fructose consumed, as human increased fructose consumption has coincided with substantial increases in caloric consumption, primarily due to increases in carbohydrate consumption (Marriott et al., 2009). In addition, these estimates in humans are based on self-reported dietary recall, which typically underestimates consumption, particularly in obese subjects and adolescents, with obese adults underreporting energy intake by an average 47% and adolescents underreporting by an average 20% (Schoeller, 1995).

It should also be noted that the high-fructose diet used in these studies contained a lower percentage fat (10%) than the chow diet (13%) as well as a substantially lower percentage fat than the average 33% fat of the American diet {Wright, 2010 #5167}. The fructose diet used was intended to explore the effects of this simple sugar, and not to mimic the American diet, which has undergone several shifts over the past decades in terms of fat and carbohydrate consumption. Between the 1970s and 2000, there was a relative increase in carbohydrate consumption and relative decrease in fat consumption, with an overall total caloric increase {Centers for Disease, 2004 #5170}, while carbohydrate consumption has dropped somewhat in the 2000s and fat intake has plateaued {Wright, 2010 #5167}. Importantly, as mentioned in the interlude, fructose by itself has lipogenic effects; short-term fructose consumption increases de novo lipogenesis, while chronic consumption is associated with dyslipidemia and ectopic lipid deposition (Tappy & Le, 2010). Moreover, fructose and fat consumption may synergize to exacerbate the effects of one another; leptin resistance induced by high-fructose feeding can promote increased weight gain after a transition to a high-fat diet in rats {Shapiro, 2008 #5171}. Fructose and fat may also act through different mechanisms to promote glucose intolerance, potentially creating an interactive effect when consumed together {Huang, 2004 #5172}.

The 55% high-fructose diet administered to the rats in these experiments was not a reflection of the typical American diet. Instead, the diet's effects should be understood in the context of exploring potential effects of a given macronutrient (fructose) on energy homeostasis and stress response, and not as a replica of the human condition. In animal models in nutrition research, it is critical to remember that mammalian basal metabolic rates (BMR) vary in proportion to mass (M) in the form BMR ~ $aM^{2/3}$ (White & Seymour, 2003). Thus, while a 60kg human may have a BMR approximately 34 times greater than a 300g rat, her BMR per unit of mass is only 17% that of the rat; and this is only one of many metabolic differences between rodents and humans (Baker, 2008). While animal models can be useful for understanding nutrient

interactions, nutrient bioavailability, and nutrient tolerance for excessive intake, careful consideration must be made before directly translating findings into humans (Baker, 2008).

9.3.2 Metabolic & behavioral effects of a high fructose diet In Chapter Five, we demonstrated that a high-fructose diet initiated in adolescence increased caloric efficiency, elevated fasting glucose, and increased peri-renal and epididymal fat pad mass in adulthood (**Figure 5.2**). Animals fed the high fructose diet in adulthood only were not susceptible to these same effects. In addition, the high-fructose diet attenuated the reduction in weight gain during chronic adolescent stress, and increased fasting glucose across the stress period. This fructose-induced weight maintenance was similarly observed in Chapter Eight after surgery (**Figure 8.2**), and could be related to increased caloric efficiency that would promote weight gain or increased adipose tissue that would provide an additional source of stored energy. In addition, the high-fructose diet altered expression of the cerebral metabolic marker GLUT1 in response to acute stress (**Figure 5.6**). Chow-fed rats significantly increased hippocampal GLUT1 expression, as would be expected based on results in Chapter Two, but stressed and non-stressed periadolescent animals had reduced GLUT1 gene expression after both the glucose challenge and the forced swim test, potentially indicating an impaired ability to respond to an energetic stressor. This lack of change in GLUT1 could be related to changes in glucocorticoid signaling, as well. Periadolescent high-fructose rats had elevated basal plasma corticosterone (**Figure 5.7**). While the attenuation in plasma corticosterone after the forced swim was not significant in periadolescent rats, it is possible that impaired glucocorticoid signaling was a mediating factor in the altered GLUT1 response to stress, given that GLUT1 is a key downstream target of the GR. A time course evaluation of the response to stress or a dexamethasone suppression test could help test this hypothesis.

Affective behaviors were also altered in the periadolescent cohort of rats fed the high-fructose diet, but not in the cohort of rats fed only during adulthood. In Chapter Five, periadolescent rats displayed increased anxiety –like and depressive-like behaviors in the elevated

plus maze and forced swim test (**Figures 5.4 and 5.5**). In Chapter Eight, periadolescent rats also displayed depressive- and anxiety-like behavior with reduced grooming in the open field and increased latency to interact in the social interaction test (**Figure 8.9**). While the behavioral effects did not perfectly match between the different cohorts, they consistently pointed toward altered affective type behaviors, which are commonly associated with disruptions of the HPA axis (Pariante & Lightman, 2008) as well as hippocampal plasticity (B. S. McEwen, 1999). Elevated corticosterone in the context of the high-fructose diet points to excess HPA axis drive, and subsequent chronic action of glucocorticoids on the hippocampus could lead to dendritic atrophy and synaptic reorganization (B. S. McEwen, 1999). In Chapter Eight, we observed increases in cerebral vasculature in the CA1 region of the hippocampus (**Figure 8.4**), which could reflect an adaptive response to counteract glucocorticoid-mediated toxicity in the context of hyperglycemia (Beauquis et al., 2010). Subsequent research should investigate the extent to which changes in plasma corticosterone and/or changes in cerebral vasculature are necessary for behavioral alterations.

In addition, evidence from Chapter Seven indicates possible alterations in the mesolimbic dopamine system, most notably with respect to hypothalamic gene expression of dopamine synthesis and signaling factors. Though these changes did not have a pronounced effect on plasma prolactin (**Figure 7.2**) or protein expression in the ventral tegmental area, nucleus accumbens, and hypothalamus (**Figure 7.4**), the study lacked the proper design to explore changes in dopamine release or turnover. In light of the abundant research relating simple carbohydrate consumption to alterations in reward circuitry (Avena, Bocarsly, et al., 2008; Hajnal et al., 2004; Meguid et al., 2000), future investigators may wish to explore the effects of a high fructose diet on motivated behavior. Studies could range from simple water deprivation tests to more complex assessments such as a drug reinforcement paradigm.

9.3.3 Effects of fructose on the hypothalamic transcriptome Consumption of a periadolescent high-fructose diet induced extensive remodeling of the hypothalamic

transcriptome. Using the CuffDiff algorithm to identify differentially expressed transcripts, we detected 966 transcripts that were differentially expressed at a False Discovery Rate with adjusted p-values <0.05, representing approximately 5.6% of the 17,366 transcripts that mapped onto known genes (Chapter Five). Pathway analysis indicated that POMC processing was the pathway most highly enriched with differentially expressed genes (**Figure 5.9**). Hypothalamic POMC, specifically within the arcuate nucleus, is best known for its role in promoting satiety via signals relayed to the paraventricular nucleus (PVN) and lateral hypothalamus (LH, (Schwartz et al., 2000)). POMC is also a precursor for ACTH and melanocyte stimulating hormone (MSH). ACTH release from the pituitary is an essential component of the HPA axis, and MSH acts on melanocortin receptors, notably MC4R (also upregulated in fructose fed animals) to inhibit feeding (Williams et al., 2001). POMC and MC4R knockout mice exhibit hyperphagia and weight gain, and increases in ACTH and MC4R are associated with increased anxiety- and depressive-like behaviors (Ryan et al., 2014; Serova et al., 2013). The extensive changes in POMC signaling represent potential targets for understanding the relationship between the metabolic and behavioral effects of the high-fructose diet.

Of course, the changes in the hypothalamic transcriptome extended beyond the POMC pathway, and the size of the dataset derived from RNA-sequencing enables multiple analytic approached to assess these changes. In Chapter Six, we used a more exploratory approach to RNA-sequencing analysis to enable greater breadth of scope. As described in O'Connor et al (O'Connor et al., 2014), differential expression was identified as transcripts in fructose-fed rats either exceeding a 1.25 or inferior to a 0.75 fold change in expression relative to chow-fed rats. No statistical testing was applied at the level of individual genes in RNA-seq and the list of differentially expressed transcripts were used to serve as an intermediary input for higher-order bioinformatics that have their own FDR adjusted p-values less than 0.05. This analysis confirmed again that POMC processing was again the pathway most highly enriched with differentially

expressed genes (**Table 6.3**). However, it also identified enrichment in multiple pathways related to the immune response, particularly the three complement pathways.

Subsequent PCR validation of specific targets in these pathways in two separate periadolescent fructose-fed cohorts confirmed increases in hypothalamic *C4b* and hippocampal *Cfb* (**Figure 6.5**). Changes in complement expression were not present in animals that consumed the high-fructose diet during adulthood only. In addition, while there were no group differences in hippocampal expression of synaptic markers, complement component gene expression and plasma corticosterone were predictive of synaptophysin and PSD95 expression in linear regression modeling (**Table 6.4 and 6.5**). Specifically, high *Cfb* and high *C4b* expression were associated with high PSD95 expression, which is consistent with the literature demonstrating colocalization of complement factors with PSD95 (Stephan et al., 2012; Stevens et al., 2007). These studies have indicated that C1q and C3 contribute to synaptic pruning and synaptic stripping, and may be related to the growing literature linking increased complement expression in neurologic disease (Eikelenboom et al., 1989; Ingram et al., 2012; McGeer et al., 1989; E. D. Pedersen et al., 2004). Next steps in this research include analysis of the cell-type specific expression of complement expression in fructose-fed animals, investigation of changes in dendritic morphology, and analysis of changes long-term potentiation or depression in hippocampal slice recordings from fructose- and chow-fed rats. In addition, alternative behavioral testing to assess learning & memory behavior might be used, but such tests should be chosen carefully as anxiety-like behavior can be a confound in several tests, e.g., Morris Water Maze and Barnes Maze (Harrison, Hosseini, & McDonald, 2009). The 8-arm radial arm maze could be a less anxiogenic test, but the reward used (typically food pellets) would need to be adjusted; in-depth testing of novel object versus novel location testing may be simpler to perform (Whishaw & Kolb, 2004). Subsequently, small molecule targeting to inhibit expression of specific complement components could help elucidate the specific role complement plays in

fructose-mediated changes in physiology and behavior. Such small molecules and antibodies exist to inhibit or deplete C1q, C3, C5, Factor B (Ricklin & Lambris, 2007).

In Chapter Seven, we took a more data-driven approach to RNA-sequencing analysis, using Principal Component Analysis to drive our understanding of the patterns within the data set. The first three PCs in this analysis accounted for 49.5% of the total variance, and fructose- and chow-fed animals clustered separately when mapped back on the PCs (Figure 7.1). Two genes related to dopamine synthesis and signaling, namely, the genes for tyrosine hydroxylase and the dopamine transporter, were in the ten highest ranking top-loading factors for PC1, indicating their importance in driving expression patterns in the dataset. Ensuing data-mining revealed significant changes in multiple genes related to dopamine synthesis and signaling. Plasma prolactin, under inhibitory control from hypothalamic dopamine, trended downward in fructose fed rats, and prolactin levels predicted hypothalamic gene expression of TH, DAT, and VMAT2. However, changes at the level of protein expression were not observed in a separate set of rats, not exposed to the same fasting challenge. Differences in dopaminergic responses between the fasting and fed state have been previously recognized with the literature (Meguid et al., 2000) and these findings highlight the importance of challenges to energy homeostasis in driving hypothalamic signals. The possibility that fructose-fed rats may differ in their response to fasting, specifically within the mesolimbic dopamine system, is an intriguing one that warrants further investigation.

9.3.4 Energy homeostasis and HPA axis effects on outcomes after ischemic injury In Chapter Eight, we sought to determine whether a high-fructose diet would prime the system for a "second hit" in the form of middle cerebral artery occlusion (MCAO)-induced ischemic injury. We hypothesized that the periadolescent high-fructose diet would exacerbate outcomes after MCAO, particularly with respect to anxiety-like and depressive-like behavior. Metabolic pathology such as diabetes is a known risk factor for depression (R. J. Anderson et al., 2001), stroke (Gunnell et al., 1998; Reilly & Kelly, 2011), and post-stroke depression (Tanislav et al.,

2015). Modeling this relationship with the high-fructose diet could highlight mechanisms leading to improved understanding of the unique etiology of post-stroke depression.

Contrary to our hypothesis, the high fructose diet did not alter neurological behavior after MCAO (**Figures 8.7 and 8.8**), nor did was fructose-induced depressive-like behavior exacerbated after stroke (**Figure 8.9**). Lesion volume also did not differ between chow- and fructose-fed MCAO cohorts. However, fructose did increase hippocampal *Vegfa* expression as well as CA1 vascular length (**Figure 8.5**). Such neovascularization can be adaptive and potentially protective effect in the context of stroke, but potentially result in greater risk of hemorrhagic transformation (W. Li et al., 2010). Additionally, future studies may wish to examine alternative outcomes after MCAO, such as changes in neuroinflammation and particularly complement expression, given the findings in Chapter Six and the established detrimental effects of complement upregulation (Engstrom et al., 2007; E. D. Pedersen et al., 2004).

9.3.5 Future directions in the study of the effects of a high-fructose diet on the stress response Specific suggestions for future study have been discussed in the previous sections as well as in each chapter throughout this thesis. However, a critical question remains regarding the mechanism whereby a high-fructose diet might mediate such widespread effects on behavior, endocrine responses, and hypothalamic remodeling. To what extent does fructose itself influence these effects, and to what extent are they a result of fructose-mediated effects on metabolism? An interesting first pass analysis could examine whether the quantity of fructose itself is altered in either CSF or cerebral tissue. Fructose can be measured in CSF by gas chromatography-mass spectrometry or liquid chromatography-Fourier transform mass-spectrometry (Wishart, 2008). Nuclear magnetic resonance imaging with ^{13}C-labeled precursors can be used to detect glucose metabolism and its metabolites, including lactate, in the rat brain *in vivo*(de Graaf, Mason, Patel, Behar, & Rothman, 2003), and could be adapted to detect fructose metabolism for comparison of cerebral glucose and fructose metabolism in fructose- and chow-fed rats. If changes in glucose and/or fructose metabolism were found, future work could seek to rectify these changes by

targeting specific parts of the metabolic pathways affected.

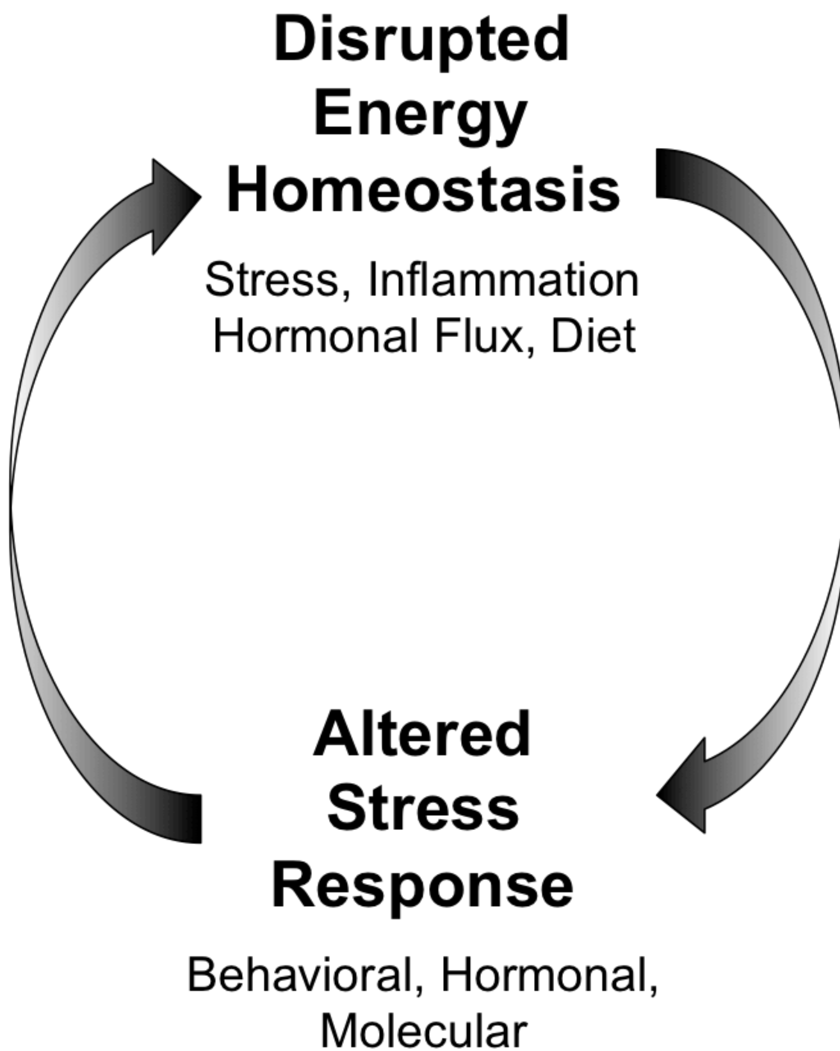
Additional methods to further disentangle the contributions of fructose versus its metabolic effects include pair-feeding; comparing the high-fructose diet's effects to a diet or genetic model that induces similar metabolic abnormalities; or by pharmacologically inducing hyperglycemia or genetically inducing obesity. Continuing studies should also seek to determine whether (and which of) the effects of the periadolescent high-fructose diet are reversible. The evidence supporting reversibility of diet or metabolic dysfunction after consumption during a critical stage is mixed. Though weaning onto a chow diet after exposure to a maternal high-fat diet reverses body mass differences in non-stressed rats (Tamashiro et al., 2009), maternal high-fat diet can also induce epigenetic changes associated with changes in body size and insulin sensitivity lasting up to two generations (Dunn & Bale, 2009). Such studies as to the reversibility of the effects of the periadolescent high-fructose diet may shed light into the mechanism whereby the diet mediates its effects.

9.4 Conclusion

Hans Selye originally described the stress response, or "general alarm reaction," as a three-stage response to nocuous agents with widespread effects across organ systems (H. Selye, 1998). This thesis has explored multiple stressors, or demands for change, including psychosocial stress, inflammatory stimuli, hormonal fluctuations, dietary modulation, and physical injury (stroke). In considering the effects of each of these stressors, we have examined how they may impact energy homeostasis. Each of these stressors causes different energetic effects, including shifts in glucose transporters, changes in weight and caloric efficiency, and alterations in the use and storage of energy. In turn, changes in energy homeostasis have shifted the stress response. Psychosocial stress-induced changes in glucose transporters altered the responsively of these same transporters to a second stressor, LPS. Hormonally-influenced

fluctuations in glucose transporters likely affected the extent to which psychosocial stress could shift expression in females. High-fructose diet-induced metabolic dysfunction was most pronounced with the diet was introduced during an energetically demanding period of development, adolescence. This dysfunction coincided with altered affective behavior and changes in HPA axis output as well as extensive remodeling of the hypothalamic transcriptome, highlighting the profound relationship between metabolism and the stress response. The fructose-induced shift in energy homeostasis additionally altered regional cerebral vasculature, which may have been protective against further energetic insult in the form of stroke. Taken together, this thesis has demonstrated that the reciprocal relationship between energy homeostasis and the stress response is far-reaching, affecting the immune system, the endocrine system, limbic circuitry, and cerebral vasculature. Truly, as Claude Bernard wisely noted, to understand the "true significance" of this relationship, we must "draw our final conclusions only in relation to the effects in the whole."

Figure 9.1. Reciprocal relationship between energy homeostasis and the stress response



Multiple disruptions to energy homeostasis, whether through psychosocial stress, inflammatory stimuli, hormonal fluctuations, and dietary changes, have widespread effects on behavioral, hormonal, and molecular outcomes.

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