2017

Examining the Effects of Adolescent Binge Alcohol Exposure on the Negative Feedback of the Hpa Axis and Adult Responses to Psychological Stress

Audrey Rose Torcaso
Loyola University Chicago, audrey.torcaso@gmail.com

Recommended Citation
Torcaso, Audrey Rose, "Examining the Effects of Adolescent Binge Alcohol Exposure on the Negative Feedback of the Hpa Axis and Adult Responses to Psychological Stress" (2017). Dissertations. 2864.
https://ecommons.luc.edu/luc_diss/2864

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.
Copyright © 2017 Audrey Rose Torcaso
LOYOLA UNIVERSITY CHICAGO

EXAMINING THE EFFECTS OF ADOLESCENT BINGE ALCOHOL EXPOSURE ON THE NEGATIVE FEEDBACK OF THE HPA AXIS AND ADULT RESPONSES TO PSYCHOLOGICAL STRESS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY PROGRAM IN NEUROSCIENCE

BY AUDREY TORCASO

CHICAGO, IL
DECEMBER 2017
ACKNOWLEDGMENTS

I would like to thank everyone who helped make this dissertation possible. First and foremost, I must thank my mentor, Dr. Toni Pak. You are not just an amazing mentor, but also an outstanding scientist, teacher, and an all-around incredible woman. You have shaped me into the scientist I am today and have exposed me to ideas and experiences that I would never encountered if not for you. None of this work would have been possible without you, and I could not have asked for a better person to guide me through this process. Words will never be able to fully express how thankful I am to you.

I would also like to thank my dissertation committee members: chair of the committee Dr. Pieter de Tombe, Dr. Pamela Witte, Dr. Michael Collins, and Dr. Miriam Domowicz. Thank you all for your critical feedback and insight. You pushed me to become the best scientist I could be and continually offered excellent advice and support. I am so grateful that you invested your time and efforts into my professional development.

Thank you to the past and present members of the Pak lab for being fantastic coworkers. You always helped keep the atmosphere light and fun, even when my experiments failed for the millionth time. Our coffee chats helped keep me grounded through everything, and I will always treasure the memories of our shenanigans.

I am also thankful for the members of the Neuroscience program, the Alcohol Research Program, and the Cell and Molecular Physiology department. You have all offered me some sort of support that I could not have done without, whether that was asking helpful questions during seminars or simply showing me how to use the printer.

Finally, I would like to thank my family and friends for molding me into who I am and sticking with me through everything. Thank you to my parents; I literally would not be here
without you, nor would I have the drive and passion for life if not for having you both as examples. Thank you to my parents-in-law; you treated me as one of your own from the beginning and having your support has helped so much. Thank you to my husband, Joe, for always providing support and helping to keep things interesting. Your love and care means so much to me.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS ix

ABSTRACT xiv

CHAPTER I: STATEMENT OF THE PROBLEM 1

CHAPTER II: ADOLESCENT BINGE ALCOHOL AND THE HPA AXIS: A REVIEW OF THE LITERATURE 4

Literature Review 4

Adolescence: A critical period of brain development and behavioral transitions 5
Adolescent binge drinking: impact in human studies and animal models 8
The hypothalamo-pituitary-adrenal (HPA) axis in health and disease 10
The glucocorticoid receptor – structure and function 13
Glucocorticoid receptor protein:protein interactions 15
Glucocorticoid feedback inhibition in the hypothalamus 20
Effects of adolescent binge alcohol on HPA axis function 21

Summary 24
Hypothesis and Aims 25

CHAPTER III: THE EFFECTS OF ADOLESCENT BINGE ALCOHOL EXPOSURE ON THE BEHAVIORAL AND NEUROENDOCRINE RESPONSES TO AN ACUTE PSYCHOLOGICAL STRESSOR 29

Introduction 29

Results 32

Discussion 43

CHAPTER IV: THE EFFECTS OF ADOLESCENT BINGE ALCOHOL EXPOSURE ON THE BEHAVIORAL AND NEUROENDOCRINE RESPONSES TO CHRONIC STRESSORS 50

Introduction 50

Results 52

Discussion 57
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Elevated plus maze trial 1 results</td>
<td>34</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Statistical analysis for elevated plus maze trial 2 and biochemical parameters</td>
<td>40</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Proteins identified in both BioID-GR samples</td>
<td>81</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Proteins identified in only BioID-GR vehicle samples</td>
<td>82</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Proteins identified in only BioID-GR dexamethasone samples</td>
<td>83</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. HPA axis schematic 10
Figure 2. GR protein:protein interactions in a PVN parvocellular neuron 16
Figure 3. Experimental paradigm 32
Figure 4. Anxiety-like behavior under resting conditions 35
Figure 5. Anxiety-like behavior following acute restraint stress 38
Figure 6. Regional levels of CRF, AVP, and cFos transcripts 41
Figure 7. Plasma CORT and brain region expression of GR 43
Figure 8. Experimental paradigm 52
Figure 9. Anxiety-like behavior following adolescent binge alcohol and either adult binge alcohol or adult repeated restraint stress 54
Figure 10. Regional levels of CRF, AVP, and GR transcripts 56
Figure 11. Effects of repeated ETOH or DEX on GR nuclear localization 65
Figure 12. GR-HSP90 interaction in the dorsal hippocampus after adolescent binge alcohol exposure 67
Figure 13. GR-HSP90 interaction in the hypothalamus after adolescent binge alcohol exposure 69
Figure 14. Hypothalamic levels of PER1 and FKBP5 70
Figure 15. Validation of BioID-GR function 79
Figure 16. PANTHER protein class analysis of BioID-GR associated proteins 85
Figure 17. STRING analysis of BioID-GR associated proteins

Figure 18. Graphical summary of key findings

Figure 19. Dot plots depicting anxiety-like behavior under resting conditions

Figure 20. Dot plots depicting anxiety-like behavior following acute restraint stress

Figure 21. Dot plots depicting regional levels of CRF, AVP, and cFos transcripts

Figure 22. Dot plots depicting plasma CORT and brain region expression of GR

Figure 23. Dot plots depicting anxiety-like behavior following adolescent binge alcohol and either adult binge alcohol or adult repeated restraint stress

Figure 24. Dot plots depicting regional levels of CRF, AVP, and GR transcripts

Figure 25. Dot plot depicting effects of repeated ETOH or DEX on GR nuclear localization

Figure 26. Dot plots depicting GR-HSP90 interaction in the dorsal hippocampus after adolescent binge alcohol exposure

Figure 27. Dot plots depicting GR-HSP90 interaction in the hypothalamus after adolescent binge alcohol exposure

Figure 28. Dot plots depicting hypothalamic levels of PER1 and FKBP5
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AF1/2</td>
<td>activation factor ½</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BAC</td>
<td>blood alcohol concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CDK5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRFR</td>
<td>corticotropin-releasing hormone receptor</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CTL</td>
<td>control</td>
</tr>
<tr>
<td>CyP</td>
<td>cyclophilin</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>D.Hipp</td>
<td>dorsal hippocampus</td>
</tr>
<tr>
<td>DMC</td>
<td>differentially methylated cytosine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DnMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DSM-IV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, 4th Edition</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>Hip</td>
<td>hsp70-interacting protein</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamo-pituitary-adrenal</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP/hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HVAC</td>
<td>heating, ventilation and air conditioning</td>
</tr>
<tr>
<td>Hypo</td>
<td>hypothalamus</td>
</tr>
<tr>
<td>i.g.</td>
<td>intragastric</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography – tandem mass spectrometry</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MeCP2</td>
<td>methyl CpG binding protein 2</td>
</tr>
<tr>
<td>MR</td>
<td>mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>nBAF</td>
<td>neuron-specific chromatin remodeling complex</td>
</tr>
<tr>
<td>NCoA</td>
<td>nuclear receptor co-activator</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nGRE</td>
<td>negative glucocorticoid response binding element</td>
</tr>
<tr>
<td>NIAAA</td>
<td>National Institute of Alcohol Abuse and Alcoholism</td>
</tr>
<tr>
<td>npBAF</td>
<td>neural progenitors-specific chromatin remodeling complex</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>PER1</td>
<td>Period 1</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PND</td>
<td>post-natal day</td>
</tr>
<tr>
<td>PP</td>
<td>protein phosphatase</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAP</td>
<td>stretched attend postures</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEGRAM</td>
<td>selective glucocorticoid receptor agonist/modulator</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SILAC</td>
<td>stable isotope labeling with amino acids in cell culture</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of the retinoic acid receptor and thyroid receptor</td>
</tr>
<tr>
<td>SRC</td>
<td>steroid receptor coactivator</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TMT</td>
<td>tandem mass tag</td>
</tr>
<tr>
<td>V.Hipp</td>
<td>ventral hippocampus</td>
</tr>
</tbody>
</table>
ABSTRACT

Binge drinking during adolescence is a common occurrence which is associated with increased risk of developing alcohol dependence and other mental health disorders. Hypothalamo-pituitary-adrenal (HPA) axis dysfunction is one characteristic commonly observed in many affective disorders, including anxiety and depression. Our laboratory has previously demonstrated that adolescent binge-pattern alcohol exposure results in long-term dysfunction of the HPA axis in a Wistar rat model, characterized by deficient glucocorticoid feedback inhibition. The current study aimed to characterize the behavioral phenotype of these rats in response to psychological stress during adulthood, and furthermore sought to understand the molecular mechanisms by which adolescent binge alcohol exposure may induce changes in HPA axis function and behavior.

To address the behavioral consequences of adolescent binge alcohol exposure, Wistar rats were exposed to our laboratory’s established adolescent binge alcohol paradigm, then subject to further acute or chronic psychological stress during young adulthood. The anxiety behaviors of these rats were measured using the elevated plus maze, then tissues were collected to assess HPA axis effector levels. To address the mechanisms by which alcohol may alter HPA axis signaling, glucocorticoid receptor (GR) co-immunoprecipitation experiments were performed using brain tissue samples from adolescent binge alcohol exposed Wistar rats. Additionally, a proximity-dependent biotinylation (BioID) screen was established to identify novel GR protein:protein interactions in a neuroblastoma-derived cell line.
The data demonstrated that adolescent binge alcohol exposure in combination with either acute or chronic adulthood stress resulted in increased anxiety-like behaviors, accompanied by HPA axis dysfunction. Adolescent binge alcohol exposure selectively altered HPA-related GR target gene expression, but not other target genes; furthermore, adolescent binge alcohol exposure did not alter GR interactions with its chaperone hsp90. Together, these data suggest alcohol specifically alters GR-mediated HPA axis regulation, but not global GR signaling. The BioID screen preliminarily identified 59 putative GR-interacting proteins, the majority of which were novel potential protein:protein interactions. Further work is needed to assess whether alcohol may affect these novel interactions. Collectively, the work presented here contributes important information regarding the effects of adolescent binge alcohol exposure at both behavioral and molecular levels.
CHAPTER I

STATEMENT OF THE PROBLEM

Adolescence is a critical period of brain development which is concomitant with a behavioral transition from being dependent on one’s parents to becoming an independent person. One behavioral hallmark of this transition is an increased propensity for experimentation with novel stimuli, including alcohol. Therefore, it comes as no surprise that binge drinking is a common behavior among adolescents; roughly half of high school seniors admit to having been drunk at some point in their lives, and roughly 5.4 million teenagers in the U.S. can be classified as “binge drinkers,” meaning they have consumed five or more alcoholic beverages in one sitting at least once a month (Johnston, O’Malley, Miech, Bachman, & Schulenberg, 2017; Results from the 2013 NSDUH: Summary of National Findings, 2014).

Because of the active developmental processes occurring during adolescence, the adolescent brain is uniquely vulnerable to alcohol exposure. Consequently, teenage binge drinking can have effects that last long past intoxication, even into adulthood, regardless of whether an individual continues to consume alcohol regularly. For example, imaging studies conducted on the brains of teenage binge drinkers reveal disorganization and thinning of brain regions that are important for regulating memories, mood, and cognition (Luciana, Collins, Muetzel, & Lim, 2013), while epidemiological studies demonstrate that teenage binge drinking increases the risk for alcohol dependence and mood disorders (Grant, Stinson, & Harford, 2001; Viner & Taylor, 2007). A considerable number of research studies using animal models of adolescent binge alcohol have
also been conducted, especially in recent years, which collectively support a causative role for adolescent binge drinking in the development of these mental health disorders. Yet, the results of specific studies can sometimes seem contradictory among one another due to differences in the animal model used (mouse, rat, non-human primate, etc.), the dosage, timing, and route of alcohol administration, and the research tools used to measure the study outcomes.

One characteristic observed in many mood disorders is dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis. Functionally, the HPA axis comprises the neuroendocrine stress response and culminates with increased release of glucocorticoids (GCs) from the adrenal cortex into the systemic circulation. GCs are pleiotropic steroid hormones, known mostly for their ability to regulate immune responses and glucose homeostasis, but they also exert negative feedback on the hypothalamic arm of the axis by downregulating expression of corticotropin releasing factor (CRF) via the glucocorticoid receptor (GR). Although HPA axis dysfunction is observed in mood disorder patients, current drug therapies for these disorders are not directly targeted toward restoring normal HPA axis function specifically. While many mood disorders first appear during the adolescent transition period, the etiologies of mood disorders are poorly understood, and many patients do not respond to drug treatments that are currently available.

Alcohol has long been known to act as a stressor, activating the HPA axis and eliciting a neuroendocrine stress response. It was not until more recently, though, that it became clear that alcohol can modulate the reactivity of the HPA axis, and in adolescence, binge pattern alcohol exposure can result in long-term HPA axis dysfunction, specifically chronically elevated hypothalamic CRF levels and an exacerbated increase in circulating GCs upon exposure to a subsequent stressor (Przybycien-Szymanska, Mott, Paul, Gillespie, & Pak, 2011). These data suggest
that alcohol is reducing the GC-mediated negative feedback on the hypothalamus, but little is known regarding how adolescent binge alcohol might be affecting hypothalamic control of the neuroendocrine stress response. *In vitro* evidence demonstrates that binge level alcohol increases CRF promoter activity and decreases the ability of GR to bind to the CRF promoter (Przybycien-Szymanska, Mott, & Pak, 2011), suggesting that alcohol’s effects on HPA axis function are mediated by GR, but how specifically alcohol may be doing this is unclear.

Collectively, there is much work to be done in understanding how mood disorders develop, how they may be effectively treated, how alcohol may impact mood disorder development, how alcohol disrupts the neuroendocrine stress response, and how alcohol’s effects on the HPA axis might alter mood and behavior, particularly in the vulnerable adolescent brain. This dissertation aims to characterize the long-term behavioral and neuroendocrine effects of adolescent binge alcohol exposure, and to understand how binge-level alcohol impacts hypothalamic glucocorticoid receptor (GR) signaling, adding to our collective knowledge regarding how adolescent binge alcohol may cause an increased propensity toward the development of a mood disorder.
CHAPTER II

ADOLESCENT BINGE ALCOHOL AND THE HPA AXIS: A REVIEW OF THE LITERATURE

Literature Review.

Binge drinking is a common behavior among adolescents that many teens and parents alike consider benign, even “normal.” Although adolescents do normally exhibit increased interest in stimulatory or risky behaviors, mounting evidence demonstrates that teenage binge drinking is a risk factor for many mental health disorders, suggesting that this behavior is, in fact, a serious public health concern (Grant et al., 2001; Rose, Winter, Viken, & Kaprio, 2014; Viner & Taylor, 2007). Because it is difficult to establish a causal relationship between teenage binge drinking and mental health disorders in human studies, many animal models have been designed that collectively demonstrate an increased propensity toward addiction and anxiety-like and/or depressive phenotypes due to adolescent alcohol exposure (Briones & Woods, 2013a; Guerri & Pascual, 2010; Pandey, Sakharkar, Tang, & Zhang, 2015). This is likely due to the fact that the brain undergoes important developmental changes during puberty, generally characterized by widespread synaptic pruning and increases in myelination (Luciana, 2013). Like the rest of the brain, the hypothalamus (and therefore reactivity of the hypothalamo-pituitary-adrenal, or HPA, axis) undergoes important developmental changes during adolescence, and perturbation of this developmental process by external stressors can result in permanent dysfunction of this neuroendocrine stress response (Romeo et al., 2006; Sisk & Zehr, 2005). Importantly, HPA axis...
dysfunction is observed in many mood disorders (Naughton, Dinan, & Scott, 2014), and our laboratory and others have demonstrated using animal models that adolescent binge alcohol exposure results in long term dysfunction of the HPA axis (Brunell & Spear, 2005; Logrip et al., 2013; Przybycien-Szymanska, Mott, Paul, et al., 2011). Taken together, these findings suggest that understanding adolescent alcohol’s effects on the HPA axis may be the key to understanding how adolescent alcohol increases the propensity toward mood disorder development. However, the molecular mechanisms by which the HPA axis is fine-tuned are poorly understood, particularly at the level of the hypothalamus, and likely involve many pathways with varied temporal resolution. This review of the literature will therefore focus on the developmental changes occurring in the adolescent brain, the effects of adolescent alcohol exposure, and the molecular regulation of the HPA axis.

Adolescence: A critical period of brain development and behavioral transitions

Adolescence is the developmental period between childhood and adulthood during which important physical and cognitive changes occur to allow an individual to transition into an independent lifestyle. While the terms “puberty” and “adolescence” are often used interchangeably, they refer to different yet overlapping developmental periods. Puberty specifically refers to the developmental time period during which an animal becomes reproductively competent, and is marked by elevated secretion of gonadal steroid hormones that results in the development of secondary sex characteristics (i.e. in humans, breast development in females, or the onset of facial hair growth in males). Indeed, the dynamic changes in steroid hormone concentrations during puberty play a role in the characteristic reorganization of the adolescent brain (reviewed in Sisk & Zehr, 2005), but the adolescent timeframe is more protracted than the strict pubertal time period. Because the adolescent timeframe can be difficult to define in non-human animals, many an-
imal models of “adolescence” are based around the onset of puberty, which can be clearly deli-
eated by structural and hormonal characteristics.

In addition to the visible physical changes that accompany puberty, non-invasive imaging
studies over the past ~25 years have allowed for characterization of the structural changes occur-
ring in the brain throughout the entire adolescent transition (extending even into the mid-twenties
in humans). In general, gray matter volume follows an inverted U-shaped trajectory, reaching
peak volume in the cortex just prior to the onset of puberty, whereas the peak subcortical gray
matter volume is delayed by a few years (Giedd et al., 2015). These decreases in gray matter
volume across adolescence are believed to be due mostly to synaptic pruning (Huttenlocher,
1990; Whitford et al., 2007). On the other hand, white matter volume, which reflects the degree
of myelination, increases linearly throughout puberty, accompanied by increased organization of
white matter tracts and therefore increased connectivity among brain regions (Giedd et al., 2015).
Additionally, these changes occur generally in caudal-to-rostral and phylogenetically older-to-
newer pattern, such that the prefrontal cortex, which is involved in higher order cognitive pro-
cesses, is the last region to fully develop (Gogtay et al., 2004).

One of the most prominent behavioral characteristics of adolescence is poor decision
making, specifically the propensity for adolescents to engage in novel, stimulating, and risky ac-
tivities. These observations, combined with the advent of adolescent neuroimaging studies, ulti-
mately led to the “dual systems” model of adolescent brain development, which states that the
incentive-processing socioemotional brain pathways mature before the inhibitory cognitive con-
trol pathways develop (Casey, Getz, & Galvan, 2008; Steinberg, 2008). While this model is like-
ly a bit of an oversimplification, it is generally supported by both psychological and neuroimag-
ing data (Mills, Goddings, Clasen, Giedd, & Blakemore, 2014; Shulman et al., 2016). Further-
more, adolescents exhibit increased responsiveness to positively rewarding stimuli and decreased responsiveness to aversive stimuli, an observation that is supported by both human and animal data (reviewed in Spear, 2011). It is therefore not surprising that the recreational use and abuse of both alcohol and illicit drugs begins on average during the early teen years, increasing throughout adolescence and peaking in the mid-twenties (Johnston et al., 2017).

Another important aspect regarding adolescent brain development is the fact that many neuropsychiatric disorders have an average onset during the adolescent timeframe. In America, the median age of onset for any DSM-IV disorder is 14 years of age, and about half of the population will be diagnosed with a DSM-IV disorder at some point in their life, highlighting the sociological burden that psychiatric disorders place on our country (Kessler et al., 2005). In regard to substance abuse disorders specifically, individuals who engage in drug or alcohol use during adolescence have an increased risk of subsequently developing a substance abuse disorder (Wagner & Anthony, 2002). Animal studies suggest this may be due in part to adolescents’ relative resistance to the negative aspects of alcohol intoxication, including motor impairments and “hangover” symptoms (Doremus, Brunell, Varlinskaya, & Spear, 2003; White et al., 2002), while at the same time, adolescents are more susceptible to the alcohol neurotoxic effects, particularly in the hippocampus (White & Swartzwelder, 2004). Likewise, affective disorders also have a typical onset during puberty, and in girls specifically, depression and anxiety onset correlate better with menarche than with chronological age, strongly suggesting the involvement of steroid hormones in the development of mood disorders (Patton et al., 1996). In summary, adolescence is a developmental period during which the brain is uniquely susceptible to perturbations from external stimuli, which can result in long-lasting structural and functional effects.
**Adolescent binge drinking: impact in human studies and animal models**

“Binge drinking” is defined by the National Institute of Alcohol Abuse and Alcoholism (NIAAA) as consuming enough alcohol to raise an individual’s blood alcohol concentration to 0.08 grams percent or higher; typically these levels are achieved when a man consumes five alcoholic beverages or a woman consumes four alcoholic beverages in the span of two hours (Alcoholism, 2004). By their senior year of high school, roughly half of teenagers will have been drunk at some point in their lives; furthermore, over 15% of 12th graders self-report frequent binge drinking (Johnston et al., 2017). In 2010, underage drinking cost the United States $24.3 billion, demonstrating the vast economic impact that teenage binge drinking has on our society (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015). Additionally, teenage binge drinking is known to increase the risk of alcoholism and mood disorders later in life (Grant et al., 2001; Rose et al., 2014; Viner & Taylor, 2007).

Human brain imaging studies have revealed many structural consequences to teenage binge drinking. Teenage binge drinkers exhibit decreased fractional anisotropy, a measure of white matter integrity and organization, throughout frontal, temporal, parietal, and cerebellar regions, suggesting widespread deficits in connectivity among brain areas, particularly within the limbic system (McQueeny et al., 2009). Similar observations have been made in longitudinal studies that compared brain scans before and after the onset of alcohol use in adolescents, particularly demonstrating decreased white matter organization among brain regions responsible for behavioral regulation and executive function; this same study observed decreases in middle frontal gyrus cortical thickness in adolescent alcohol users, which is also considered to be part of the cognitive control network (Luciana et al., 2013). Heavy binge drinking adolescents also exhibit greater decreases in cortical volume, which might indicate an increase in the normal synap-
tic pruning that occurs during this developmental time period, or could indicate early neuro-
degeneration (Squeglia et al., 2015). Teenage binge drinkers also exhibit physiological dysfunc-
tions; for example, female college students with higher self-reported drinking have increased sal-
ivary cortisol levels, suggesting dysregulation of the HPA axis (Wemm et al., 2013).

Because of the limitations of most human studies, it is difficult to draw causal relation-
ships between teenage binge drinking and various outcomes; therefore, many animal models of
adolescent alcohol exposure have been developed. However, there is not one single animal mod-
el of binge drinking during puberty that is standardized across this field of research; therefore, it
is important to keep in mind each experimental paradigm when interpreting a study’s results.
Male Wistar rats exposed to ethanol vapor inhalation three consecutive days per week for four
weeks beginning at post-natal day (PND) 32-34 induced deficits in spatial working memory
measured by the Morris water maze in the week after the last ethanol treatment, but did not alter
anxiety behavior tested on the elevated plus maze (Schulteis, Archer, Tapert, & Frank, 2008). A
similar ethanol-vapor delivery study observed decreased forebrain neurogenesis in the subven-
tricular zone up to 21 days after the last dose (Hansson et al., 2010), while intragastric delivery
of high doses of alcohol resulted in decreased hippocampal neurogenesis in Sprague-Dawley rats
(McClain, Hayes, Morris, & Nixon, 2011). Reduced hippocampal neurogenesis has also been
observed in male Rhesus monkeys that were trained to consume alcohol during adolescence for
11 months and then abstained from alcohol for two months (Taffe et al., 2010). Voluntary etha-
nol consumption in rats has also been shown to cause altered dopaminergic neurotransmission
and social behavior (Maldonado-Devincci, Badanich, & Kirstein, 2010). While the list of effects
due to adolescent binge alcohol exposure continues to grow, our laboratory and others have
shown that alcohol induces long-term effects on the regulation of the HPA axis (Allen, Lee,
Alcohol has long been shown to activate the HPA axis, thereby increasing circulating glucocorticoids (Adinoff, Iranmanesh, Veldhuis, & Fisher, 1998; Inder et al., 1995). However, the mechanisms by which a short-term alcohol stress can induce long-term changes to the HPA axis are unknown.

Figure 1: HPA axis schematic. Upon exposure to a stressor, the PVN of the hypothalamus (left) releases CRF and AVP. These neuropeptides act on the anterior pituitary (middle) to stimulate the release of ACTH into the systemic circulation. ACTH acts on the adrenal cortex to trigger glucocorticoid release into the circulation. Glucocorticoids exert effects throughout the body, mainly on metabolism and immune function, and exert negative feedback on the hypothalamus. Alcohol has been shown to disrupt this feedback inhibition.

The hypothalamo-pituitary-adrenal (HPA) axis in health and disease

The ability to respond appropriately to stressors is a critical aspect of all life forms. While all living organisms must respond to physical stressors (i.e. any perturbation to homeostasis) lest
they become permanently disabled or succumb to death, higher order animals have developed the
capacity to also respond to perceived threats to their safety, or psychological stressors. While the
immediate “fight-or-flight” response to stress is mediated by the sympathetic nervous system,
long term maintenance of homeostasis in the context of stress requires the integration of nervous,
endocrine, and immune system responses. This stress response is mediated by the hypothalampituitary-adrenal (HPA) axis, the activation of which has widespread physiological and psycholog-ical consequences (see Figure 1) (reviewed in Smith & Vale, 2006).

Upon exposure to a stressor, parvocellular neurons in the paraventricular nucleus (PVN)
of the hypothalamus release the neuropeptide corticotropin-releasing factor (CRF) into the hypophysial portal circulation; once CRF binds its receptor (CRFR1) on anterior pituitary corticotropes, adrenocorticotropic hormone (ACTH) is released into the systemic circulation (Rivier & Vale, 1983; G. W. Smith et al., 1998; Vale, Spiess, Rivier, & Rivier, 1981). Although vasopres-sin (AVP) is most well-known for its role in osmoregulation, AVP can be released from par-
vocellular PVN neurons onto the pituitary corticotropes as well, where binding to their V1b recep-tors potentiate ACTH release (Hernando, Schoots, Lolait, & Burbach, 2001; S. M. Smith & Vale, 2006). When ACTH reaches the adrenal cortex, it binds its receptor (MC2-R) on adreno-
cortical parenchymal cells, which triggers intracellular signaling cascades that result in increased steroidogenesis and secretion of glucocorticoids (Simpson & Waterman, 1988; S. M. Smith & Vale, 2006). Glucocorticoids (cortisol in humans and corticosterone in rodents) are pleiotropic hormones which act via binding their cognate receptors, either the glucocorticoid receptor (GR), or the mineralocorticoid receptor (MR); however, it is generally believed that MR regulates basal HPA axis tone while GR is responsible for carrying out the effects of glucocorticoids under stressed conditions (Ratka, Sutanto, Bloemers, & de Kloet, 1989; Reul & de Kloet, 1985). The
GR is a class I nuclear receptor with widespread distribution of expression throughout the brain and periphery, therefore its effects are canonically mediated by changes in target gene expression, which is highly cell-specific (Bamberger, Schulte, & Chrousos, 1996; Love et al., 2017). Generally, glucocorticoids mediate changes in metabolism, immune function, cognition, and behavior; these changes are adaptive at appropriate levels but can become pathogenic if dysregulated (Charmandari, Tsigos, & Chrousos, 2005). Thus, activity of the HPA axis is under tight regulation by both neuronal and endocrine signals (Figure 1). Glucocorticoids themselves exert negative feedback on both the hypothalamus and pituitary, and because GR is widely distributed throughout the brain, both the parvocellular PVN neurons themselves as well as other brain regions, including the hippocampus, are potential sites for glucocorticoid feedback inhibition (Jacobson & Sapolsky, 1991; Sawchenko, 1987). Furthermore, other neuronal populations throughout the hypothalamus, brainstem, lamina terminalis, and limbic system extend projections to the parvocellular PVN neurons to regulate HPA axis activity in response to a variety of different types of stressors (S. M. Smith & Vale, 2006).

Although many physical disorders are associated with a hyper- or hypo-active HPA axis, including diabetes, suppressed immune function, gastrointestinal issues, decreased reproductive function, and inhibition of thyroid function (reviewed in Charmandari et al., 2005), we will focus this portion of the review of the literature on psychiatric disorders associated with HPA axis dysfunction due to the relevance to the work at hand. The most common mood disorder, major depression, is associated with HPA axis dysfunction characterized by increased central CRF levels, downregulation of CRF receptors, enhanced adrenal responses to ACTH, and downregulation of glucocorticoid feedback inhibition (Naughton et al., 2014). A low-dose dexamethasone suppression test has been proposed as a diagnostic tool for depression (Carroll et al., 1981), but its use
has not become widespread due to low specificity of the results (i.e., a number of other factors can alter responsiveness to this test). Furthermore, although drugs targeting the HPA axis, such as cortisol synthesis inhibitors, have been proposed as therapeutic interventions for depression, these drugs are not favored, as they are not sufficient to achieve remission on their own (Wolkowitz & Reus, 1999). HPA axis dysfunction is also associated with anxiety disorders, but the specific dysfunction can vary based on the type of anxiety disorder. For example, patients with panic disorder exhibit normal responsiveness to dexamethasone, but their cortisol response is hypo-reactive to CRF injection (Petrowski, Wintermann, Kirschbaum, & Bornstein, 2012). In contrast to depressed patients, those with PTSD actually exhibit enhanced glucocorticoid feedback inhibition (Yehuda, Yang, Buchsbaum, & Golier, 2006). Interestingly, patients with co-morbid PTSD and major depression exhibit a distinct HPA axis profile, with an attenuated ACTH response and low morning CORT levels, suggesting dysregulation at the level of the hypothalamus (de Kloet et al., 2008; Vythilingam et al., 2010). Together, these studies highlight the importance of characterizing the specific mechanisms responsible for mood disorder-associated neuroendocrine dysfunction and the need for better HPA axis targeted therapeutics.

The glucocorticoid receptor – structure and function

The glucocorticoid receptor (GR) is a ligand-activated transcription factor that is part of the nuclear receptor superfamily. Encoded by the NR3C1 gene, in humans the mature protein is 777 amino acids long and is composed of three major functional domains: the N-terminal transactivation domain (NTD), the DNA-binding domain (DBD), and a multifunctional C-terminal domain (CTD) which contains the ligand binding site, a second transactivation domain, nuclear localization signals, and sites for other proteins to bind (Giguère, Hollenberg, Rosenfeld, & Evans, 1986). An additional nuclear localization signal is located in a flexible hinge region of the
protein between the DBD and CTD, and perhaps not surprisingly based on the proximity to the ligand binding site, nuclear localization is hormone dependent (Picard & Yamamoto, 1987).

The NTD is the most variable domain in GR, both among the various nuclear receptor superfamily members and among different species (Kumar & Thompson, 1999). This domain contains the AF1 transcription activation domain, which can act as a docking site for the transcriptional machinery and coregulatory proteins (Dieken & Miesfeld, 1992). The NTD also contains all of the known phosphorylation sites; some of these sites are phosphorylated upon ligand binding, and each site’s phosphorylation has different effects on GR’s capability to modulate transcription (W. Chen et al., 2008; Pocuca, Ruzdijic, Demonacos, Kanazir, & Krstic-Demonacos, 1998; Wang, Frederick, & Garabedian, 2002). Another post-translational modification in this region is SUMOylation at K293, which has been demonstrated to be necessary for the formation of a complex required for tethered transrepression important for anti-inflammatory processes (Hua, Ganti, & Chambon, 2015; Hua, Paulen, & Chambon, 2016). Together, these data highlight the importance of this domain in the fine-tuning of GR signaling.

The DBD, like other nuclear receptors, contains two highly conserved zinc fingers which can bind a particular DNA sequence referred to as a glucocorticoid response element, or GRE (Nicolaides, Galata, Kino, Chrousos, & Charmandari, 2010). Though the complete absence of GR is neonatally lethal, expression of a mutant GR (containing a point mutation within the DBD) which cannot directly bind DNA is not necessary for survival, though these mutant GR-expressing mice do exhibit abnormalities in the hypothalamus and pituitary (Reichardt et al., 1998). Interestingly, recent studies have demonstrated that variability in the DNA sequence where GR binds can confer conformational shifts within the GR DBD that can alter its dimerization, suggesting that GR-DNA signaling is a “two-way street” (Watson et al., 2013).
Finally, the CTD contains many functional regions, including the ligand binding domain, the AF2 transactivation region, one of the nuclear localization signals, and binding sites for receptor dimerization and coregulatory and chaperone proteins (Beck, De Bosscher, & Haegeman, 2011; Bledsoe et al., 2002). Some naturally occurring, disease-linked mutations occur in the GR CTD, some of which render the receptor unable to bind ligand, and others which simply destabilize the protein (Bledsoe et al., 2002). Interestingly, crystal structures reveal that binding of the antagonist mifepristone causes a conformational shift in helix 12 (part of the LBD) that favors corepressor binding, demonstrating how different ligands can induce GR conformations that favor one effect on transcription (based on coregulatory protein binding) over another (Frego & Davidson, 2006; Pfaff & Fletterick, 2010; Schoch et al., 2010).

In humans, there are two major isoforms of GR that arise from an alternative exon 9, resulting in a different and slightly shorter CTD in the GRβ isoform (Hollenberg et al., 1985). Though GRβ is generally thought of as a dominant-negative form of the receptor, it also exhibits intrinsic, gene-specific transcriptional activity in a ligand- and GRα-independent manner (Kino et al., 2009; Oakley, Jewell, Yudt, Bofetiado, & Cidlowski, 1999). Mice also express a different GRβ isoform that is also truncated at the C-terminus, but a GRβ isoform has not been characterized in rats. All three organisms, however, have an alternative translation start site, which can produce slight truncations at the N-terminus. Humans also express several different untranslated exon 1 variants that may regulate receptor protein levels (Turner & Muller, 2005).

Glucocorticoid receptor protein:protein interactions

The glucocorticoid receptor carries out its effects in conjunction with many other proteins. This includes, but is certainly not limited to, a suite of proteins that bind to GR to stabilize it in the cytoplasm, proteins that facilitate transfer into (and possibly out of) the nucleus, and tran-
scriptional coregulatory proteins. These interactions are likely somewhat cell-type specific, and changes in GR conformation due to ligand binding and/or DNA binding can expose various surfaces through which GR can interact with its binding partners. Therefore, fully understanding these protein:protein interactions is part of the key to understanding GR function as a whole.

**Figure 2: GR protein:protein interactions in a PVN parvocellular neuron.** GR is localized in the cytoplasm in the absence of ligand, where it remains in a complex with heat shock proteins and other co-chaperones, as well as other binding partners like FKBP5. During stress, adenylyl cyclase activity increases, which activates cAMP-PKA-CREB signaling, increasing CRF expression. When glucocorticoid levels rise, GR translocates into the nucleus where it downregulates CRF expression.

In the absence of ligand, GR is predominantly localized in the cytoplasm of the cell, where it resides in a multimolecular complex which holds the receptor in a conformation with high affinity for ligand. Nascent GR is first bound to hsp70 and hsp40, which then facilitate
hsp90 binding; this creates what is sometimes referred to as the foldosome (Morishima, Murphy, Li, Sanchez, & Pratt, 2000; Rexin, Busch, & Gehring, 1991). Additional co-chaperone proteins which assist in this process may involve hsp70-interacting protein (Hip) or BAG family molecular chaperone regulator 1 (BAG-1) (Kanelakis et al., 2000). In order for GR to mature, p23 can bind the GR-hsp90 complex in order for the GR ligand binding domain to open into a conformation that will allow hormone binding (Morishima et al., 2003). To complete this maturation process, immunophilins including FK506-binding proteins FKBP51 or FKBP52, cyclophilin-40 (CyP-40), and protein phosphatase 5 (PP5) bind the complex (Cheung & Smith, 2000). Interestingly, FKBP51 and FKBP52 are believed to play opposing roles on GR function by altering its affinity for hormone; FKBP51 downregulates GR activity, while FKBP52 increases it (Riggs et al., 2003).

Although canonically it was thought that ligand binding resulted in GR’s dissociation from this cytoplasmic chaperone complex, recent data suggest that this complex actually facilitates GR nuclear translocation. For example, treatment of cells with the hsp90 inhibitor geldanamycin slows down GR nuclear translocation (Elbi et al., 2004; Galigniana et al., 1998). Also, FKBP52 coimmunoprecipitates with GR, hsp90, and the motor protein dynein (Silverstein et al., 1999), demonstrating how this complex would facilitate a more rapid translocation mechanism via the cytoskeleton. Once at the nuclear envelope, GR entry into the nucleus is mediated by importins recognizing and binding the nuclear localization signals on GR (Freedman & Yamamoto, 2004). Recent data demonstrated that members of the GR-hsp90 complex can also interact with importins and a nuclear pore glycoprotein (Nup62), raising the question that this chaperone complex might also translocate with GR (Echeverria et al., 2009).
Once inside the nucleus, GR’s interactions with other proteins ultimately mediates its effects on transcription; it is important, however, to note that most studies which investigate GR protein:protein interactions are conducted in the context of immune cell (or another non-neuronal cell type) function. It is well known that GR’s ultimate target gene regulation is cell-type specific (Krstic, Rogatsky, Yamamoto, & Garabedian, 1997; Love et al., 2017), suggesting that GR’s nuclear protein:protein interactions could also vary from cell to cell. Like other members of the nuclear receptor family, GR requires recruitment of coregulatory proteins to influence transcription (Petta et al., 2016). In order to exert transactivation of target genes, GR may recruit a variety of coactivators, the most well-studied of which is the p160 family, comprised of NCoA1 to -3 (also known as SRC-1 to -3 or by other individual names) (Anzick et al., 1997; Hong, Kohli, Trivedi, Johnson, & Stallcup, 1996; Oñate, Tsai, Tsai, & O’Malley, 1995). These p160 family coactivators interact with nuclear receptors (including GR) via their highly conserved LXXLL motifs (Parker, Heery, Kalkhoven, & Hoare, 1997). These coactivators then recruit histone acetyltransferases, which alter chromatin formation into a more open, transcriptionally active state (Li, Wong, Tsai, Tsai, & O’Malley, 2003). Of particular note, despite being generally referred to as a “coactivator,” NCoA2 can in some contexts act to repress transcription rather than activate it (Rogatsky, Zaremba, & Yamamoto, 2001). On the other hand, nuclear receptors can also interact with corepressors (NCoRs) and a homologue SMRT (silencing mediator of the retinoic acid receptor and thyroid receptor) which interact with the nuclear receptors via a L/I-XXI/V-I motif (Collingwood, Urnov, & Wolffe, 1999). Not surprisingly, these corepressors can exert opposite effects on chromatin formation by recruiting histone deacetylases (Rosenfeld, Lunyak, & Glass, 2006). The GR antagonist mifepristone (RU486) does not inhibit GR from binding DNA; rather,
it triggers the recruitment of corepressors in scenarios where GR would normally recruit coactivators (Schulz et al., 2002).

GR can also interfere with other transcription factor signaling pathways. For example, GR and NF-κB can directly bind one another, resulting in mutual inhibition of their actions on downstream target genes (Ray & Prefontaine, 1994); additionally GR and NF-κB signaling can intersect via several other mechanisms (Petta et al., 2016). Similarly, GR and AP-1 can inhibit one another, although recent evidence has demonstrated that AP-1 is also necessary for GR-mediated transcription of some target genes (Biddie et al., 2011; Yang-Yen et al., 1990). Another bidirectional regulatory relationship exists between GR and the Jak/STAT signaling pathway; GR can interfere with the Jak/STAT signaling pathway by directly binding to some of its mediators, while STAT3 can affect the transcription of GR and other GR-binding proteins (de Miguel, Lee, Onate, & Gao, 2003; Langlais et al., 2012).

Export of GR from the nucleus can also occur, and it is generally believed that out of the total population of GR in a cell, some of it is constantly in flux between nucleus and cytoplasm; the presence or absence of ligand simply tilts that balance in one direction or another. GR nuclear export is mediated by the nuclear export receptor calreticulin, though it is possible that this is not the only exportin that can remove GR from the nucleus (Olkku & Mahonen, 2009; Walther et al., 2003). While it is generally believed that GR is degraded after nuclear export, it has recently been proposed that exported GR, in conjunction with hsp90, may be recycled instead (DeFranco, 2000).

GR can undergo post-translational modifications in ways which are relevant to its function. For example, S203 and S221 become hyperphosphorylated by CDK5 in the presence of glucocorticoids, while S226 phosphorylation by JNK negatively regulates GR activity (Ismaili &
Garabedian, 2004; Rogatsky, Logan, & Garabedian, 1998). Furthermore, GR can be tagged for degradation by ubiquitination (Duma, Jewell, & Cidlowski, 2006), and it can be SUMOylated such that it can form a transrepression complex (Hua et al., 2015). Certainly, GR’s interactions with the enzymes that mediate these post-translational modifications are also important to its function in the cell.

Glucocorticoid feedback inhibition in the hypothalamus

While glucocorticoids can mediate feedback inhibition of the HPA axis at both the hypothalamic and pituitary levels (reviewed in Laryea, Muglia, Arnett, & Muglia, 2015), the exact molecular mechanisms by which this occurs is largely understudied. Here, we will focus specifically on glucocorticoid negative feedback mechanisms in the parvocellular PVN neurons. Originally, it was demonstrated using deletions of the CRF promoter in gel shift and reporter assays that a so-called negative glucocorticoid response element (nGRE) exists within the CRF promoter, and that GR can bind this region primarily as a monomer, suggesting that negative feedback occurred via direct GR-DNA binding at the CRF promoter region, which could then interfere with AP-1 or CREB-mediated increases in CRF expression (Malkoski & Dorin, 1999). However, subsequent studies have opposed this original model; for example, mice that express a mutant GR with a point mutation within the DBD that renders it unable to bind DNA do not show changes in CRF expression, suggesting direct DNA binding is not necessary for GR to regulate CRF expression (Reichardt et al., 1998). Furthermore, ChIP assays demonstrate relatively little interaction of GR with the CRF promoter relative to other transcription factors at the CRF promoter, or GR binding at other promoter regions, suggesting this mechanism of feedback inhibition is minor (Evans, Liu, MacGregor, Huang, & Aguilera, 2013).
Other proposed mechanisms for glucocorticoid mediated negative feedback include interactions with other signaling pathways. For example, experiments using the BE(2)C neuroblastoma cell line suggest GR might exert repression of CRF expression by interacting with and interfering with the activity of PKA (which would alter the ability of other transcription factors like CREB to increase CRF promoter activity); however this study did not demonstrate a role of histone deacetylases (HDACs) in altering CRF expression (Yamamori et al., 2007). This is in opposition to more recent studies that have demonstrated, using the PVN-derived IVB cell line, that DEX treatment results in formation of a repressor complex including GR, HDAC1, methyl CpG binding protein 2 (MeCP2), and DNA methyltransferase 3b (DnMT3b), which together result in epigenetic changes in the CRF promoter region, including methylation of the promoter (Sharma, Bhave, Gregg, & Uht, 2013). These modifications likely would not explain all glucocorticoid mediated feedback inhibition, as some inhibition is observed on a much shorter timescale. Other have demonstrated that glucocorticoids can increase endocannabinoid signaling, thereby reducing the frequency of excitatory glutamatergic stimuli from other brain regions, which could be mediated by a putative membrane GR or could be due to glucocorticoid binding to other membrane receptors (Di, Malcher-Lopes, Halmos, & Tasker, 2003; Evanson, Tasker, Hill, Hillard, & Herman, 2010). Together, these studies demonstrate that there are likely several mechanisms which regulate glucocorticoid mediated feedback inhibition of CRF, and resolving each mechanism on a molecular scale with relevant temporal resolution is important for understanding the complexities of HPA axis regulation as a whole.

Effects of adolescent binge alcohol on HPA axis function

Our laboratory has demonstrated that in male Wistar rats, binge alcohol exposure during puberty results in HPA axis dysregulation that persists into adulthood but that binge alcohol ex-
posure during adulthood results in different effects on the HPA axis than what is seen in adolescence (Przybycien-Szymanska, Mott, Paul, et al., 2011; Przybycien-Szymanska, Rao, & Pak, 2010). The choice to use Wistar rats specifically is important because they are an outbred strain, which is more reflective of the human population, and they exhibit greater fluctuations in HPA axis effectors in response to stress compared to other outbred rat strains, though in behavioral anxiety tests they exhibit higher baseline anxiety-like behaviors (D’Souza El-Guindy et al., 2010; Harbuz, Jessop, Lightman, & Chowdrey, 1994; Rex, Voigt, Gustedt, Beckett, & Fink, 2004). Furthermore, Wistar rats are widely used throughout the stress literature, which gives a better frame of reference for comparison.

Specifically, our laboratory has demonstrated that a single dose of 3.0g/kg EtOH results in elevation of circulating CORT levels in both pubertal and adult Wistar rats; this CORT increase in response to EtOH is slightly attenuated in pubertal and adult rats after the last dose of EtOH in our binge paradigm, but is still elevated compared to control animals. However, in male pubertal rats, CRF and AVP expression in the PVN is increased ONLY in response to binge EtOH exposure, but not control or acute alcohol exposure. Female rats do have higher baseline CRF and AVP expression levels, but these levels do not change in response to acute or binge EtOH treatment (Przybycien-Szymanska et al., 2010). These sexual dimorphisms are due to the presence of 17β-estradiol, but interestingly, ovariectomized female rats exposed to adolescent binge alcohol exhibit decreases in CRF and AVP expression (Przybycien-Szymanska, Gillespie, & Pak, 2012). Furthermore, male rats that were exposed to binge alcohol during puberty and subsequently treated with a second acute- or binge- alcohol challenge exhibit altered HPA responsiveness to this second treatment. Pre-exposed adult male rats had lower baseline CORT levels than EtOH-naïve adult rats, but increases in circulating CORT in the pre-exposed rats after
a single EtOH dose exceeded the increases observed in naïve rats exposed to a single EtOH dose. Additionally, pre-exposed rats did not have an attenuated CORT increase when exposed to binge alcohol in adulthood, but the naïve rats did have an attenuated CORT release. Our study indicated that pre-exposed male rats had higher baseline CRF expression in the PVN than naïve rats, which increased after acute but not binge EtOH treatment, while AVP expression in the PVN of pre-exposed male rats showed a completely different pattern compared to naïve rats (Przybycien-Szymanska, Mott, Paul, et al., 2011). Our lab has investigated the possible mechanisms underlying this HPA axis dysfunction using a PVN-derived cell line (IVB), demonstrating that two hours of treatment with alcohol doses as low as 12.5mM resulted in increased CRF promoter activity in a luciferase assay; this is likely due to the inability of GR to bind the CRF promoter, as observed by ChIP assays (Przybycien-Szymanska, Mott, & Pak, 2011). Together, these data demonstrate that adolescent alcohol exposure in a Wistar rat model results in hypersensitivity of the HPA axis caused by dysfunctional feedback inhibition by glucocorticoids at the level of the hypothalamus.

Other studies investigating adolescent alcohol’s effects on the HPA axis generally agree with our laboratory’s findings, but some results may be mixed, likely due to differences in experimental paradigm. For example, adolescent Sprague-Dawley rats (which are derived from the Wistar rat strain but exhibit slight differences in stress responsiveness) when given access to alcohol during a period of daily foot-shock stress do not habituate to the stressor, supporting the idea that adolescent alcohol interferes with HPA axis negative feedback and plasticity (Brunell & Spear, 2005). On the other hand, Sprague-Dawley rats exposed to alcohol vapor from PND28-42 did not exhibit significant increases in PVN CRF levels as adults, but males did show increased AVP expression in the PVN on PND42 (Logrip et al., 2013). Interestingly, adolescent alcohol
administration has been shown to alter histone modifications, specifically observed in the amygdala but likely also in other brain regions, which suggests adolescent alcohol use can alter the epigenetic landscape in potentially heritable ways (Pandey et al., 2015). Indeed, our laboratory has observed changes in hypothalamic gene expression profiles of offspring born to parents with a history of adolescent binge alcohol exposure (Przybycien-Szymanska, Rao, Prins, & Pak, 2014). Although we have not directly studied the role of histone modifications in this alteration of gene expression patterns, we have demonstrated that these offspring exhibit differential cytosine methylation across the genome, which explains some but not all of the observed changes in hypothalamic gene expression (Asimes et al., 2017).

**Summary.**

Adolescent binge alcohol exposure results in hypersensitivity of the HPA axis due to disrupted feedback inhibition by glucocorticoids. This dysfunction may underlie a propensity toward the development of mood disorders in teenage binge drinkers, and the fact that the adolescent brain undergoes widespread developmental changes makes this a unique time period during which exposure of the brain to any perturbation can have permanent consequences. Several potential mechanisms by which glucocorticoids mediate feedback inhibition at the level of the hypothalamus have been described, making it unclear how alcohol might disrupt this process. In general, glucocorticoids act via their receptor, GR, which is a ligand-dependent transcription factor that works in conjunction with many other proteins. The fact that alcohol does not change the levels of GR itself suggests that it may influence GR protein:protein interactions, which can vary in different cell types. However, the neural GR interactome is vastly under studied. It is important to understand the molecular mechanisms by which adolescent alcohol exposure causes
HPA axis hypersensitivity and an increased risk of mood disorders in order to guide the development of novel therapeutics that prevent or treat the effects of teen binge drinking.

**Hypothesis and Aims.**

Since the HPA axis comprises the major neuroendocrine stress response, and dysfunction of the HPA axis is associated with many psychiatric disorders, it is important to understand how adolescent binge alcohol consumption exerts its effects on the HPA axis. The goal of the current research, then, is to elucidate the underlying molecular mechanisms mediating adolescent binge alcohol’s effects on the GR (and therefore on the HPA axis), as well as investigate the neuroendocrine and behavioral responses to psychological stress in adult animals that were exposed to binge alcohol during adolescence. The overarching hypothesis of this work is that binge alcohol during adolescence alters the GR’s interactions with other proteins, resulting in increased CRH expression and aberrant HPA axis signaling, which contributes to inappropriate neuroendocrine and behavioral responses to psychological stress during adulthood. To test this hypothesis, I have developed the following aims:

**Aim 1: Evaluate the behavioral and neuroendocrine responses to psychological stress in adulthood following adolescent binge alcohol exposure in a rat model.**

Many animal studies have aimed to test the causal link between adolescent binge alcohol exposure and an anxiety-like phenotype, but the results have been difficult to interpret due to differences in experimental paradigms, highlighting the importance of evaluating each one experimentally. While our lab has established a connection between adolescent alcohol and long-term HPA axis dysfunction in a particular peri-pubertal Wistar rat model, we had not previously tested whether this model would result in altered anxiety-like behavior after a prolonged abstinence from alcohol. Additionally, the development of mood disorders like anxiety tends to progress
due to a variety of compounded factors or multiple stressful events. It was unknown how rats exposed to binge alcohol during adolescence would respond to subsequent stressors, and if those responses varied depending on whether the subsequent stressor was acute versus repeated or homotypic versus heterotypic. Therefore, I sought to answer the following questions:

1) Is adolescent binge alcohol exposure alone sufficient to cause an anxiety-like behavioral phenotype in young adulthood?

2) Does adolescent binge alcohol exposure change the behavioral response to an acute psychological (heterotypic) stressor during adulthood?

3) Is the HPA axis profile different in rats with a history of adolescent binge alcohol exposure, with or without subsequent acute exposure to psychological stress?

4) Does adolescent binge alcohol exposure combined with repeated stress during young adulthood result in an anxiety-like phenotype?

5) After adolescent binge alcohol exposure, do homotypic and heterotypic repeated stressors result in the same behavioral and neuroendocrine responses?

Overall, the data collected in this aim demonstrated that adolescent binge alcohol altered the behavioral and neuroendocrine responses to an acute psychological stressor toward an anxiety-like phenotype, although adolescent binge alcohol alone is not sufficient to result in increases in anxiety-like behavior after long-term alcohol abstinence. Furthermore, repeated exposure to both a homotypic and heterotypic stressor during adulthood had similar effects on anxiety-like behavior and HPA axis effector levels in rats exposed to adolescent binge alcohol.

Aim 2: Characterize the mechanisms by which adolescent binge alcohol alters GR function.

Previous in vitro and in vivo studies in our lab have collectively demonstrated that binge alcohol
reduces the ability of GR to negatively regulate CRF expression, but it is still unclear how exactly this occurs. Others have demonstrated that alcohol exposure during a different developmental time period (gestation) results in region-specific changes in GR nuclear localization, suggesting alcohol may alter the suite of proteins that interact with GR in the cytoplasm. However, most studies investigating how GR regulates its target genes are performed in non-neuronal cells, and it has been demonstrated that GR target genes are cell-type specific. There are relatively few studies which have aimed to understand how GR regulates CRF gene expression in the PVN, thereby controlling HPA axis negative feedback. Therefore, it is also necessary to understand how GR functions normally in PVN neurons before one can fully understand how alcohol disrupts GR function in these cells. Therefore, in this aim I have attempted to answer the following questions:

1) Does GR interact differently with known binding partners in brain tissue punches from rats exposed to binge alcohol during adolescence?

2) Is the regulation of GR target genes intact in brain tissue punches from adolescent alcohol exposed rats?

3) Given the same amount of ligand, does alcohol change GR nuclear localization in vitro?

4) What proteins interact with GR in neuronal-like cells in the absence and presence of ligand?

In this aim, I have demonstrated that adolescent binge alcohol does not alter the association of GR with HSP90 (a chaperone protein that canonically interacts with GR in the cytoplasm) in a region-specific manner. Furthermore, some but not all GR target genes tested are unchanged in the alcohol exposed samples despite differences in glucocorticoid levels. Repeated alcohol exposure may subtly reduce the ligand-induced nuclear localization of GR in a PVN-derived cell line,
but this is likely not the sole mechanism by which adolescent alcohol exerts its effects. Finally, we described known and novel components of the putative GR interactome in a neuron-like cell line. We identified several components of the nBAF complex using a GR-directed proximity-dependent biotinylation screen, most of which had previously been shown to interact with GR. Furthermore, we identified many other nuclear proteins known to act as transcriptional coregulators or modulators of splicing that had not previously been demonstrated to be GR-interacting proteins.
CHAPTER III
THE EFFECTS OF ADOLESCENT BINGE ALCOHOL EXPOSURE ON THE BEHAVIORAL AND NEUROENDOCRINE RESPONSES TO AN ACUTE PSYCHOLOGICAL STRESSOR

Introduction.

Alcohol consumption has long been known to act as a stressor on the HPA axis, ultimately resulting in increased circulating glucocorticoids in both humans and animal models (Adinoff et al., 1998; Inder et al., 1995). Because widespread developmental changes occur in the adolescent brain, the possibility of alcohol consumption itself and alcohol-induced HPA axis activity to negatively impact normal adolescent brain development has become a recent topic of investigation. Epidemiological data have demonstrated that individuals with a history of teenage binge drinking have an increased risk of developing mental health disorders, including alcohol use disorders and mood disorders like anxiety or depression (McCambridge, McAlaney, & Rowe, 2011; Rose et al., 2014; Viner & Taylor, 2007). Similarly, studies utilizing rodent models of adolescent binge alcohol exposure have sometimes reported increases in anxiety-like behaviors after adolescent alcohol, but the data can vary depending on the specific animal model used, and the mechanism for why this occurs is poorly understood. Our laboratory has adopted a relatively prudent animal model of adolescent binge alcohol exposure, and we have demonstrated that this model results in long-term dysfunction of the HPA axis in males (Przybycien-Szymanska, Mott, Paul, et al., 2011; Przybycien-Szymanska et al., 2010). However, the behavioral phenotype of animals in
this experimental paradigm had not been characterized previously, which was a critical gap in our knowledge regarding this model’s applicability to human health. Because HPA axis dysfunction is a characteristic observed in mood disorder patients (Naughton et al., 2014), I hypothesized that male rats exposed to our adolescent binge alcohol paradigm would exhibit increased anxiety-like behaviors compared to water-treated controls. Our previous studies showed that a subsequent stressor was necessary to detect differences between alcohol- and water-exposed animals’ plasma CORT levels (Przybycien-Szymanska, Mott, Paul, et al., 2011). Therefore, it was possible that changes in anxiety-like behaviors would similarly only be detected after activation of the HPA axis by a stressful stimulus.

Because there is no single animal model of adolescent binge drinking that is uniformly used for research, there are conflicting reports on the possible causal role of adolescent binge alcohol consumption to produce an anxiety-like phenotype. Multiple studies utilizing adolescent Sprague-Dawley rats have demonstrated an increase in anxiety-like behaviors in rats exposed to binge-level alcohol either via self-administration, or i.p. injections (Briones & Woods, 2013a; Pandey et al., 2015). However, male Wistar rats exposed to binge-level alcohol via vapor inhalation during adolescence did not exhibit increases in anxiety-like behavior on the elevated plus maze (Schulteis et al., 2008). Likewise, C57BL/6 mice given i.p. alcohol injections during adolescence do not exhibit increase anxiety-like behaviors (Coleman, He, Lee, Styner, & Crews, 2011). Together, these studies highlight the discrepancies in results obtained via different experimental paradigms, emphasizing the need to validate each model of adolescent binge alcohol exposure before attempting to extend the findings to the human population.

Our model of adolescent binge alcohol exposure, in which Wistar rats are given binge-level (3g/kg) alcohol via oral gavage during peri-puberty (PND37-44) for a total of six non-
consecutive days, has been shown to cause long-term dysfunction of the HPA axis in males (Przybycien-Szymanska, Mott, Paul, et al., 2011). Specifically, while CRF transcript levels are consistently elevated in the PVN of the binge-alcohol exposed animals (regardless of further treatment), plasma CORT levels appear “normal” at rest, but the CORT response elicited after a subsequent alcohol dose is intensified, suggesting the HPA axis is hypersensitive to stress due to ineffective negative feedback of glucocorticoids in the PVN. Similar HPA axis dysfunction is not observed if the rats are exposed to binge alcohol during adulthood, highlighting adolescence as a distinct developmental timeframe during which HPA function is vulnerable to permanent perturbations. Hypersensitivity of the HPA axis is also observed clinically in patients with anxiety and depression (Naughton et al., 2014). HPA dysfunction is common to many mood disorders, and the onset of mood disorders is often observed during adolescence (Paus, Keshavan, & Giedd, 2008); this finding raises the intriguing thought that HPA axis dysfunction may lie at the heart of the adolescent alcohol-induced propensity toward the development of a mental health disorder.

Here, our aim was to assess the effects of a heterotypic stressor (acute restraint stress) on the neuroendocrine stress response in young adult rats which had been exposed to our paradigm of repeated binge alcohol during adolescence. Furthermore, we measured anxiety-like and related ethological behaviors in these animals as adults in order to further validate our model’s similarity to clinical observations of increased mood disorder risk in populations who have engaged in teenage binge drinking. This was the first study to conduct these experiments in this specific animal model of adolescent binge alcohol exposure, and was an important step in confirming this model as an effective tool to study the mechanisms of alcohol’s effect on the neuroendocrine stress response system. I hypothesized that male Wistar rats exposed to our adolescent binge-pattern alcohol paradigm would exhibit increased anxiety-like behaviors in the elevated-plus
maze after subsequent exposure to an acute psychological stressor during adulthood. We demonstrated that young adult animals exposed to our paradigm of binge-level alcohol administration during adolescence that received acute restraint prior to behavioral testing did exhibit increased risk assessment behaviors and had an altered neuroendocrine profile. While restraint stress itself also activated the HPA axis, as was predicted, we observed no differences in behavior prior to any adulthood stress between water- and alcohol-exposed rats. Together, these data suggest that adolescent binge alcohol exposure could sensitize individuals to subsequent mild stressors and increase their risk of developing anxiety disorders as adults, underscoring the need to identify the mechanisms by which alcohol plays a causative role on HPA axis dysfunction and other alterations to anxiety-related brain functions.

Figure 3: Experimental paradigm. Pubertal binge ethanol (EtOH) treatments commenced on PND 37. On PND 73, animals were tested in the elevated plus maze (EPM) to establish a baseline level of anxiety-like behavior. On PND 74, animals in the restraint stress group were placed in a plastic restrainer tube for 30 min, then were tested in the EPM again, and 5 min after ending the EPM test, the animals were euthanized.

Results.

Adolescent binge alcohol exposure did not alter baseline anxiety-like behaviors in adulthood

Previously, we have demonstrated that adolescent binge alcohol exposure results in significant long term alterations in expression patterns of genes that regulate the neuroendocrine stress response as well as stress hormone biochemical profiles (Przybycien-Szymanska, Mott, Paul, et al., 2011). This led to the hypothesis that animals exposed to repeated binge alcohol dur-
adolescence may also exhibit altered anxiety-like behaviors as young adults, either at rest or after a subsequent, heterotypic stressor. To measure this, we used the elevated plus maze as it is a gold standard in the field for measuring anxiety-like behavior. Canonically, more time spent in the closed arms of the maze is indicative of an anxiety-like phenotype, while more time spent in the open arms of the maze is indicative of a more exploratory phenotype (Walf & Frye, 2007).

The first elevated plus maze behavioral trial was conducted after four weeks of complete alcohol abstinence and in the absence of any further stressful treatments (see Fig. 3 for experimental paradigm). The data from this behavioral trial showed that there were no significant differences in traditionally-measured anxiety-like behaviors, specifically the time spent in the closed arms, open arms, or intersection of the arms of the elevated plus maze, between rats exposed to repeated binge alcohol during adolescent versus rats that receive water control treatments (see Fig. 4A-C, Table 1). Furthermore, there were no significant differences between alcohol or water treated animals on any ethological behaviors measured, which included stretched attend postures (SAPs), head dips, or rearings (Fig. 4D-F, Table 1). Importantly, there were no differences in the overall activity levels between the two groups either, as measured by the total distance travelled in the maze and the average speed of exploration (Fig. 4G-H, Table 1). Together, these data suggest that adolescent binge alcohol alone does not change the anxiety-like behavioral phenotype of rats under low stress conditions.
<table>
<thead>
<tr>
<th>Dependent variable (units)</th>
<th>H2O mean (SEM)</th>
<th>ETOH mean (SEM)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in closed arms (%)</td>
<td>38.53 (3.735)</td>
<td>38.27 (2.577)</td>
<td>-9.446 to 8.926</td>
</tr>
<tr>
<td>Time in open arms (%)</td>
<td>35.33 (4.572)</td>
<td>33.07 (3.463)</td>
<td>-13.87 to 9.347</td>
</tr>
<tr>
<td>Time in intersection (%)</td>
<td>25.4 (1.682)</td>
<td>28.31 (1.648)</td>
<td>-1.861 to 7.673</td>
</tr>
<tr>
<td>Stretched attend postures (count)</td>
<td>19.2 (1.417)</td>
<td>19.15 (1.022)</td>
<td>-3.587 to 3.487</td>
</tr>
<tr>
<td>Head dips (count)</td>
<td>33.6 (3.646)</td>
<td>38.35 (3.75)</td>
<td>-5.838 to 15.34</td>
</tr>
<tr>
<td>Rearing (count)</td>
<td>23.3 (2.442)</td>
<td>24.9 (1.304)</td>
<td>-4.004 to 7.204</td>
</tr>
<tr>
<td>Total distance travelled (m)</td>
<td>13.93 (1.148)</td>
<td>14.66 (0.6127)</td>
<td>-1.911 to 3.358</td>
</tr>
<tr>
<td>Average speed (m/s)</td>
<td>0.049 (0.002967)</td>
<td>0.04885 (0.002023)</td>
<td>-0.00742 to 0.00712</td>
</tr>
</tbody>
</table>

**Table 1: Elevated plus maze trial 1 results.** Adolescent alcohol exposure did not significantly change any parameters measured in the elevated plus maze on PND73, prior to further manipulation. n=20 per group.
Figure 4: Anxiety-like behavior under resting conditions. The percent of time in the closed arms (A), intersection (B), and open arms (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=20 per group).
Adolescent binge alcohol exposure increased risk assessment behaviors in adulthood following an acute mild psychological stressor

In our previous studies, changes in stress hormone levels were not detected in unstressed animals that had received either repeated binge alcohol or water treatment during adolescence; it was only upon exposure to another stressor that differences in plasma corticosterone levels between adolescent treatment groups were observed (Przybycien-Szymanska, Mott, Paul, et al., 2011). Therefore, we next tested whether adolescent binge alcohol exposure would alter the behavioral response to a mild psychological stressor in young adulthood. Similarly to the baseline behavioral tests, rats that did not receive a psychological stressor immediately prior to testing did not exhibit difference in the spatiotemporal or ethological behavioral parameters between the adolescent alcohol or water treatment groups (refer to the black bars in Fig. 5). However, animals exposed to binge alcohol during adolescence spent significantly more time in the intersection of the elevated plus maze (but no changes in the time in the open or closed arms) after 30 minutes of restraint stress compared to rats who receive water treatment during adolescence and subsequently stressed before elevated plus maze testing (Fig. 5A-C, Table 2). Further, there were statistically significant main effects by two-way ANOVA of both adolescent alcohol exposure and of acute restraint stress on the number of SAPs, such that the rats exposed to binge alcohol as adolescents and then subjected to restraint stress just prior to entering the maze exhibited the highest number of SAPs (Fig. 5D, Table 2), but there were no statistically significant differences between treatment groups on the number of head dips or rearings (Fig. 5E-F, Table 2). Again, there were no differences between any of the groups on the total distance travelled or average speed of exploration in the maze, demonstrating that the changes in the abovementioned behaviors were not due to an overall increase in activity (Fig. 5G-H, Table 2). While the importance of
the increased time spent in the intersection of the maze is debated (as it may be due to a variety of factors which cannot be differentiated using the elevated plus maze alone), the increased level of the anxiety-relevant SAP behaviors is significant, because it suggests that adolescent binge alcohol (combined with acute restraint stress) contributes to an anxiety-like phenotype.
Figure 5: Anxiety-like behavior following acute restraint stress. The percent of time in the closed arms (A), open arms (B), and intersection (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There was a significant main effect of gavage on time spent in the intersection of the arms (C), and significant main effects of gavage and restraint on stretched attend postures (D). Asterisk (*) denotes significant pairwise comparisons between indicated groups.
Adolescent alcohol exposure altered neuroendocrine regulators of the HPA axis in adulthood

While our previous studies measured HPA axis effector levels in adolescent binge alcohol exposed animals after a homotypic stressor (a subsequent dose or repeated doses of alcohol) (Przybycien-Szymanska, Mott, Paul, et al., 2011), we also wanted to measure HPA axis mediators in adolescent binge alcohol exposed animals after a heterotypic stressor (30 minutes of restraint stress, a completely psychological stressor). The paraventricular nucleus (PVN) of the hypothalamus is the nucleus which contains the CRF- and AVP-positive neurons that project to the median eminence to regulate the HPA axis directly, and the ventral hippocampus (V.Hipp) is one brain region which sends projections to the PVN to modulate its activity (S. M. Smith & Vale, 2006), therefore we have focused our gene expression studies on these two brain regions. In addition to measuring CRF and AVP levels, we also measured cFos transcript levels as a proxy for general neuronal activity in these brain areas. In the V.Hipp, neither prior adolescent alcohol exposure nor restraint stress altered CRF mRNA (Fig. 6A, Table 2). Restraint stress did significantly increase CRF mRNA levels in the PVN of both alcohol exposed and control animals, as expected, but there was not a statistically significant effect of adolescent alcohol exposure, a finding that deviates from our previous work but may be due in part to the different experimental paradigm (Fig. 6C, Table 2). Restraint stress also significantly increased cFos expression in both the V.Hipp and the PVN, suggesting increased brain activity in these regions, but again alcohol did not significantly affect cFos expression in either region (Fig. 6 B,D, Table 2). Interestingly, adolescent alcohol exposure (but not restraint stress) increased AVP mRNA in the PVN (Fig. 6E, Table 2). Because AVP released from the PVN acts in concert with CRF to promote ACTH secretion from the anterior pituitary, the combination of increased AVP and similar (though not significantly higher) CRF levels in the PVN suggests that the PVN of rats exposed to adolescent
binge alcohol is primed to elicit a greater ACTH (and likely CORT) response during a stressful event, which is consistent with our previous studies demonstrating HPA axis hypersensitivity in adolescent binge alcohol exposed animals.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Interaction: Gavage x Restraint</th>
<th>Main effect: Gavage</th>
<th>Main effect: Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time in closed arms</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Time in open arms</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Time in intersection</td>
<td>NS</td>
<td>F(1,36)=9.47, p=0.0040</td>
<td>NS</td>
</tr>
<tr>
<td>Stretched attend postures</td>
<td>NS</td>
<td>F(1,36)=4.193, p=0.0479</td>
<td>F(1,36)=4.193, p=0.0479</td>
</tr>
<tr>
<td>Head dips</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rearing</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total distance travelled</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Average speed</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>V. hipp CRF</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>V. hipp cFos</td>
<td>NS</td>
<td>NS</td>
<td>F(1,36)=6.059, p=0.0188</td>
</tr>
<tr>
<td>PVN CRF</td>
<td>NS</td>
<td>NS</td>
<td>F(1,36)=6.102, p=0.0184</td>
</tr>
<tr>
<td>PVN cFos</td>
<td>NS</td>
<td>NS</td>
<td>F(1,28)=36.21, p&lt;0.0001</td>
</tr>
<tr>
<td>PVN AVP</td>
<td>NS</td>
<td>NS, F(1,36)=3.999, p=0.0531</td>
<td>NS</td>
</tr>
<tr>
<td>CORT</td>
<td>NS</td>
<td>F(1,36)=5.223, p=0.0283</td>
<td>F(1,36)=70.98, p&lt;0.0001</td>
</tr>
<tr>
<td>V. hipp GR</td>
<td>NS</td>
<td>NS</td>
<td>F(1,36)=5.234, p=0.0281</td>
</tr>
<tr>
<td>PVN GR</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2: Statistical analysis for elevated plus maze trial 2 and biochemical parameters. F and p-values from two-way ANOVA on EPM results at PND74, qRT-PCR, and ELISA.
Figure 6: Regional levels of CRF, AVP, and cFos transcripts. mRNA levels of corticotropin releasing factor (A and C), cFos (B and D), and arginine vasopressin (E), in the ventral hippocampus (A and B) and paraventricular nucleus of the hypothalamus (C-E), measured by RT-qPCR relative to the unrestrained H2O group. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There were significant main effects of restraint on V.hipp cFos (B), PVN CRF (C), and PVN cFos (D) levels. Asterisk (*) denotes significant main effect of restraint in panel B and significant pairwise comparisons between indicated groups in panels C and D.
Adolescent alcohol exposure altered circulating glucocorticoid levels, but not glucocorticoid receptor transcript levels

Finally, we measured plasma levels of the stress hormone corticosterone (CORT), as well as glucocorticoid receptor (GR) transcript levels in the V.Hipp and PVN, since it is known that CORT, an endogenous GR ligand, can downregulate GR expression (Yuan et al., 2016). As expected, plasma CORT levels were significantly higher following an acute stressor in both groups, such that alcohol and acute restraint stress increased CORT levels (Fig 7A, Table 2). Restraint stress significantly decreased GR mRNA levels in the V.Hipp; however, the animals with adolescent alcohol exposure exhibited a smaller decrease compared to unrestrained controls than the animals exposed to water gavage during adolescence (Fig. 7B, Table 2). There were no statistically significant differences in GR mRNA in the PVN among any of the groups, though the restrained water control rats trended toward a decrease compared to their unstressed counterparts (Fig. 7C, Table 2). This lack of a difference in GR expression in the PVN, particularly between the restrained and unrestrained alcohol exposed rats, is actually an intriguing result, as it suggests that there is dysfunction in the HPA axis negative feedback loop due to the reduced capability of GR to regulate its target genes in the PVN, which is consistent with our previous in vivo and in vitro studies (Przybycien-Szymanska, Mott, & Pak, 2011; Przybycien-Szymanska, Mott, Paul, et al., 2011).
Discussion.

The data presented here contribute novel information regarding the ability of adolescent binge alcohol exposure to alter risk assessment behavior and neuroendocrine function after mild psychological stress in adulthood, adding to the body of literature supporting the hypothesis that a history of adolescent binge drinking has a causative role in the development of mood disorders. Our experimental paradigm is relatively prudent compared to other models with regard to the timing and dosage of alcohol administration, the use of an outbred rat strain, and the low-stress environmental conditions under which the behavioral experiments were performed, which highlights the importance of our significant observations as being more applicable to the human population. Of note is that we did not observe significant alterations in behavior between alcohol-exposed and control rats prior to further stress, which is consistent with the notion that not all

Figure 7: Plasma CORT and brain region expression of GR. Circulating plasma levels of corticosterone (A), measured by ELISA. mRNA levels of glucocorticoid receptor in the ventral hippocampus (B), and paraventricular nucleus of the hypothalamus (C), measured by RT-qPCR relative to the unrestrained H2O group. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There were significant main effects of gavage and restraint on plasma CORT (A), and a significant main effect of restraint on V. hipp GR mRNA (B). Asterisk (*) denotes significant pairwise comparisons between indicated groups in panel A and significant main effect of restraint in panel B.
individuals who engage in binge drinking during adolescence will develop a mental health disorder. Instead, our data demonstrate that a history adolescent binge alcohol causes subtle changes in the manner in which a psychological stressor is perceived, which may render an individual more at risk for developing a mood disorder. Furthermore, these behavioral changes were accompanied by an altered HPA neuroendocrine profile, which is another hallmark of many mental health disorders.

My data have demonstrated that both prior adolescent binge alcohol and an acute adult restraint stress increased the number of stretched attend postures (SAPs), a risk assessment behavior, in the EPM immediately following restraint. We did not observe changes in the spatio-temporal measures of anxiety-like behavior in the EPM, which is consistent with data demonstrating that these behaviors do not originate from the same molecular mechanisms. Specifically, while the time spent in the open or closed arms of the EPM is sensitive to benzodiazepine treatment and one-trial tolerance, the incidence of SAPs is not (Albrechet-Souza, Cristina de Carvalho, Rodrigues Franci, & Brandão, 2007). Rather, the number of SAPs exhibited is positively correlated with circulating glucocorticoid levels and can be modulated by exogenous glucocorticoid treatment or pharmacological blockade of glucocorticoid synthesis (Mikics, Barsy, Barsvári, & Haller, 2005; Rodgers et al., 1999). Likewise, we observed concurrent increases in plasma CORT levels due to both adolescent binge alcohol and adult acute restraint stress that followed the same pattern as the increases in SAPs. Therefore, it is likely that the hormonal abnormalities induced by adolescent binge alcohol exposure underlie the behavioral changes we and others have observed, and may mediate the increased propensity for mental health disturbances.
Another interesting and unexpected behavioral observation we made was that rats exposed to adolescent binge alcohol spent more time in the intersection of the EPM arms upon their second exposure to the maze. However, there are no publications to our knowledge that have reported a change in this behavioral parameter independently, which leaves this observation open to many interpretations. One possibility is that this spatiotemporal behavior is another type of risk assessment, since the rats most often exhibited SAPs in the intersection of the maze (data not shown). Another explanation is that, since these differences were only observed upon the second exposure to the maze, it is possible the alcohol-exposed rats did not remember their prior exposure to the maze. Indeed, intermittent adolescent binge alcohol exposure has been shown to induce learning deficits in both a conditional discrimination task and a spatial working memory task (Pascual, Blanco, Cauli, Miñarro, & Guerri, 2007; Schulteis et al., 2008). However, further behavioral studies would have to be performed to delineate whether either of these two proposed explanations for the observed behavior is correct. For example, many researchers have switched to using the elevated zero maze in place of the elevated plus maze to measure anxiety-like behavior because it retains the characteristics of having open and enclosed portions in which the animal may spend time, but because of the shape, does not have an intersection between the two areas (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). Other proposed assays for learning deficits might include the Morris water maze, which tests spatial learning, or the novel object recognition task, which measures whether the animal remembers being exposed to an object previously (Antunes & Biala, 2012; Morris, 1984; Vorhees & Williams, 2006).

In terms of the neuroendocrine effects of adolescent binge alcohol consumption, we generally observed similar results as in our previous studies with some exceptions. As before, we observed that a history of adolescent binge alcohol exposure resulted in a hypersensitive CORT
response to a stressor, in this case an acute psychological stressor, whereas before we tested the CORT response to a homotypic alcohol stressor (Przybycien-Szymanska, Mott, Paul, et al., 2011). Likewise, in both studies, AVP transcript levels in the PVN were increased in adolescent binge alcohol exposed animals, although in the current study the data were just shy of what is traditionally considered significant. In the current study, however, we did not see a significant effect of alcohol exposure on levels of CRF transcript in the PVN (though there was a trend toward an increase), whereas our previous study demonstrated that adolescent binge alcohol did increase PVN CRF mRNA levels (Przybycien-Szymanska, Mott, Paul, et al., 2011). It is important to note the differences in the two experimental paradigms, however, particularly the fact that in the current study, all animals were tested in the EPM just prior to tissue collection, so it is possible that the behavioral testing alone induced a slight increase in PVN CRF mRNA levels in all experimental groups.

In the current study, we also measured other HPA axis effector levels that we had not measured previously. Importantly, we measured GR transcript levels in both the PVN and the V.Hipp (which has afferent connections to the PVN), and while we did not observe changes in PVN GR mRNA, we did see a decrease in V.Hipp GR mRNA due to restraint stress. This is an interesting observation, because GR itself is a GR-target gene, and because restraint stress increased plasma CORT levels, one would expect the transcript levels of GR in target tissues to decrease. The fact that we did not observe a decrease in PVN GR mRNA levels after stress suggests that the GR may be dysfunctional in this brain region specifically, and because the PVN is the site of HPA axis activation, this is likely the culprit for the dysfunction of the HPA axis overall.
As a proxy for general neuronal activation, we also measured cFos mRNA levels in the V.Hipp and PVN, and while restraint stress did increase cFos in both regions as expected, adolescent binge alcohol exposure did not have an effect, suggesting that the changes due to the history of alcohol exposure were not simply due to changes in overall neuronal activity. It is also important to point out that acute restraint stress did result in changes to cFos, PVN CRF, and V.Hipp GR transcript levels as well as plasma CORT levels as expected, demonstrating that this acute restraint stress was sufficient to activate the neuroendocrine stress response.

In comparison to other models of adolescent binge alcohol exposure, our model is relatively pragmatic in the timing and dosage of alcohol delivery, our choice of animal (rat) and strain, and in the manner in which the behavioral testing was conducted. We delay the onset of alcohol exposure until PND37, which has been demonstrated biochemically to be the onset of peri-puberty (Ketelslegers, Hetzel, Sherins, & Catt, 1978; Södersten, Damassa, & Smith, 1977). This is in stark contrast to most of the models in the field which begin alcohol delivery around PND28 (Coleman et al., 2011; Pandey et al., 2015), which is only one week after what is traditionally considered “weaning,” and would correspond to roughly an elementary school-aged human. On the days when rats receive alcohol in our model, it is administered three hours after “lights on,” which is the time of day when circulating CORT levels are normally low as it is toward the beginning of the rats’ sleeping period (Kalsbeek et al., 2012; Spiga, Walker, Terry, & Lightman, 2014), and corresponds to when human teenagers would most likely engage in binge drinking (i.e., when they should be going to sleep). This is in contrast to the widely used “drinking in the dark” model of adolescent binge alcohol consumption, in which animals consume alcohol during the dark period, which for rodents, is the wake-phase of the circadian period. We use rats versus mice because their alcohol metabolism is more similar to humans, and Wistar rats
specifically because they exhibit greater fluctuations in HPA axis effectors in response to stress compared to other outbred rat strains (D’Souza El-Guindy et al., 2010; Harbuz et al., 1994). However, it is important to note that Wistar rats exhibit higher baseline anxiety-like behavior in several assays, which could result in a ceiling effect once exposed to subsequent anxiogenic stimuli (Rex et al., 2004). Finally, we take great care to conduct our behavioral experiments under low-stress conditions (i.e. dim light, frequent handling by the same individual, soothing white noise), and while other researchers generally do not mention the conditions under which their behavioral testing is performed, anecdotal evidence would suggest that most researchers do not take the same kind of measures to reduce non-specific stress.

It is not surprising, then, that with so much variation in experimental methodology, there is great variation in the field regarding the ability of adolescent binge alcohol exposure to cause an anxiety-like phenotype in rodent models. For example, adolescent Sprague-Dawley rats that are trained to self-administer alcohol exhibit increased immobility in the open field test, which is indicative of anxiety-like behavior (Briones & Woods, 2013b). Likewise, Sprague-Dawley rats that were given 2g/kg of ethanol i.p. from PND28-41 in a two-day on, two-day off fashion demonstrated increased anxiety-like behaviors in both the light-dark box and EPM tasks as adults (Pandey et al., 2015). Conversely, C57BL/6 mice that were given 5 g/kg ethanol i.p. for ten days during adolescence (PND28-37) did not exhibit increased anxiety-like behaviors in the EPM or open field test (Coleman et al., 2011). Likewise, male Wistar rats exposed to ethanol vapor inhalation beginning around PND32-34 do not exhibit changes in the time spent in the open or closed arms of the EPM, similar to our observations in the current study (Schulteis et al., 2008). Taken together, these studies demonstrate the discrepancies in observed anxiety-like behavior across different models of adolescent binge alcohol exposure, which underscores the im-
importance of validating each model separately before conducting more labor-intensive mechanistic experiments. Furthermore, because our model is more pragmatic relative to those in the field, we believe our findings are applicable to a wider proportion of the human population, who may not display clinical symptoms of anxiety or depression, but are at a higher risk for developing a mood disorder under further psychosocial pressure.

Overall, this study contributes vital information regarding the impact of adolescent binge alcohol exposure on subsequent behavioral and neuroendocrine responses to a stressful stimulus in a physiologically relevant rat model. We demonstrated that rats with a history of adolescent binge alcohol exposure exhibited increased risk assessment behaviors and HPA axis hypersensitivity. We believe this is reflective of the observation among human patients suggesting that a history of teenage binge drinking contributes to an increased risk for mood disorders (McCormack et al., 2011; Rose et al., 2014; Viner & Taylor, 2007). Most importantly, we have validated this model as a relevant tool to study the molecular mechanisms by which adolescent alcohol exposure contributes to the development of psychiatric disease.
CHAPTER IV
THE EFFECTS OF ADOLESCENT BINGE ALCOHOL EXPOSURE ON THE BEHAVIORAL AND NEUROENDOCRINE RESPONSES TO CHRONIC STRESSORS

Introduction.

Persistent physical and psychological stressors are increasingly becoming prominent factors in both mental and physical health and disease. For example, chronic stress is known to increase the propensity toward developing mental health disorders (Pittenger & Duman, 2008). These data are corroborated by animal studies that used various chronic stress paradigms as models for anxiety or depression (Tamashiro, Nguyen, & Sakai, 2005; Willner, Muscat, & Papp, 1992). Not surprisingly, chronic stress can also alter HPA axis function, often in opposition to the ways acute stress affects the HPA axis (McEwen, 2008). Furthermore, while repeated exposure to the same stressor usually results in attenuation of the stress response to that homotypic stressor, repeated exposure to one stressor actually increases the neuroendocrine response to a different (heterotypic) stressor (Ma, Lightman, & Aguilera, 1999). Interestingly, our lab previously demonstrated that a history of repeated adolescent binge alcohol exposure eliminates the neuroendocrine habituation to repeated binge alcohol during adulthood (Przybycien-Szymanska, Mott, Paul, et al., 2011). However, it is unknown how repeated exposure to binge alcohol during adolescence might affect the neuroendocrine and behavioral adaptations to repeated heterotypic stressors during adulthood.

Our previous studies demonstrated that a history of adolescent binge alcohol abolished
the habituation to subsequent repeated alcohol exposure during adulthood (i.e., the CORT response is not attenuated after repeated exposures to alcohol). Therefore, we tested if a history of adolescent binge alcohol exposure would alter the adult response to repeated exposure to a repeated heterotypic stressor. Further, we assessed the effects of adolescent binge alcohol on the behavioral responses to these chronic stressors, and examined whether or not the behavioral response was similar for both the repeated homotypic (binge alcohol) and heterotypic (restraint) stressors. I hypothesized that male Wistar rats exposed to our adolescent binge-pattern alcohol paradigm would exhibit increased anxiety-like behaviors in the elevated-plus maze after subsequent exposure to both the chronic homotypic and heterotypic stressors during adulthood compared to naïve animals. In order to test this, we used the same adolescent binge alcohol paradigm as in our previous studies, but followed that with either eight days of restraint stress (30 min. per day), a second round of binge alcohol, or as a control, eight days of handling (5 min. per day) during young adulthood (see Fig. 8). Animals were evaluated for anxiety behaviors 24 hours after the last exposure to this second round of stressors (homotypical: EtOH; or heterotypical: restraint). The data showed a trend toward an anxiety-like phenotype in rats with a history of adolescent alcohol that were subsequently exposed to either of the chronic adult stressors, although these effects were not statistically significant between groups. Additionally, we observed differences in select HPA axis effectors measured 24 hours after stressor exposure. Together, these data suggest that adolescent binge alcohol exposure could sensitize individuals to subsequent chronic stressors, whether they are similar or different from stressors they have previously experienced.
Adolescent binge alcohol may alter behavioral response to a stressor regardless of the type of stress.

The studies conducted and discussed in Chapter III examined the effect of adolescent binge alcohol exposure on the responses to a heterotypic, purely psychological stressor. Our laboratory’s previous work had focused on the neuroendocrine effects of adolescent binge alcohol on the response to a homotypic stressor (alcohol), which is both a physical and psychological stressor (Przybycien-Szymanska, Mott, Paul, et al., 2011). Therefore, in order to be able to draw conclusions between these studies, we wanted to test whether adolescent binge alcohol exposure induced similar or different changes in anxiety-like behavior after exposure to a homotypic (binge alcohol) or heterotypic (repeated restraint) stressor. Additionally, in this set of experiments, the animals were only tested in the elevated plus maze once, 24 hours after the last stress exposure, to reduce the possibility that prior exposure to the maze would influence the results of the test, as has sometimes been observed (Albrechet-Souza et al., 2007). We observed some trends toward an anxiety-like phenotype in the animals with a history of adolescent binge alcohol exposure that were exposed to an adult chronic stressor, though these fell short of statistical significance. For example, adult stress increased the amount of time spent in the closed arms of the EPM ($F(2,30)=2.49$, $p=0.0999$, see Fig. 9A), particularly in the groups that received adolescent binge alcohol exposure.
binge alcohol. In contrast, rats that received water during adolescence did not spend more time in the closed arms after chronic adult stress. Reciprocally, in the adolescent alcohol groups, binge or restraint stress reduced the amount of time spent in the open arms of the EPM, but there was no change in the time spent in the intersection of the maze arms (Fig. 9B and C). Together, these suggest that a history of adolescent binge alcohol exposure in addition to any type of adult stressor combine to produce an anxiety-like phenotype. Of particular interest was that adolescent alcohol-exposed rats did not exhibit increased stretched attend postures after stress compared to adolescent alcohol-exposed, non-stressed rats. However, the adolescent water-exposed rats did decrease the number of stretched attend postures after stress, suggesting that a history of adolescent alcohol exposure results in increased (or in this case, retained) risk assessment behaviors (Fig. 9D). It also appeared as though, in the adolescent alcohol animals, that after stress, the rats exhibited fewer head dips, which are a more exploratory behavior (Fig. 9E). Again, this is in contrast to rats that received water during adolescence, as all groups exhibited roughly the same number of head dips regardless of chronic adult stress. There were no differences between adolescent water- and alcohol-treated animals on the number of rearings exhibited, except in the groups which received chronic restraint stress during adulthood (Fig. 9F). Importantly, there were no differences among any of the groups on the total distance travelled or the average speed of exploration in the maze, suggesting the observed changes in behavior were not due to overall changes in locomotor activity (Fig. 9G,H).
Figure 9: Anxiety-like behavior following adolescent binge alcohol and either adult binge alcohol or adult repeated restraint stress. The percent of time in the closed arms (A), intersection (B), and open arms (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA in which p<0.05 was considered significant (n=6 per group).
Adolescent binge alcohol may alter HPA axis effectors in response to a stressor regardless of the type of stress

Next, we measured transcript levels of HPA axis effectors as well as plasma CORT levels 24 hours after the last stressor (just following behavioral testing). There was a significant main effect of adolescent binge alcohol exposure on PVN CRF transcript levels (F(1,30)=5.989, p<0.05, Fig. 7A), though interestingly, the alcohol-exposed animals had decreased PVN CRF, which is in contrast to our laboratory’s previous findings (Przybycien-Szymanska, Mott, Paul, et al., 2011). This appears to be a region-specific change, as there was no change in CRF transcript levels in the V.Hipp due to adolescent alcohol exposure, though adolescent water-treated animals showed lower V.Hipp CRF after restraint stress only (Fig. 10B). Adolescent alcohol exposure also decreased AVP transcript levels in the PVN (F(1,30)=4.161, p=0.0503, Fig. 10C), which is at least partially consistent with our previous studies. Finally, the levels of GR mRNA in both the PVN and V.Hipp were unchanged (Fig. 10D,E), despite a trend toward increased plasma CORT in the adult stressed conditions, particularly in animals with prior adolescent alcohol exposure (F(2,30)=2.896, p=0.0708, Fig. 10F).
Figure 10: Regional levels of CRF, AVP, and GR transcripts. mRNA levels of corticotropin releasing factor (A and B), arginine vasopressin (C), and glucocorticoid receptor (D and E), in the paraventricular nucleus of the hypothalamus (A,C,E) and ventral hippocampus (B and D), measured by RT-qPCR relative to the unrestrained H2O group. Circulating plasma levels of corticosterone (F) were measured by ELISA. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=6 per group). There was a significant main effect of adolescent alcohol on PVN CRF (A).
**Discussion.**

The data presented here demonstrate that rats with a history of adolescent binge alcohol respond similarly to repeated homotypic and heterotypic stressors during adulthood. There was a trend toward an anxiety-like phenotype in rats with a history of adolescent binge alcohol in response to either type of repeated stress during adulthood. In the current study, adolescent alcohol exposure decreased PVN CRF levels, measured 24 hours after the last stress exposure and immediately after behavioral testing. There was also a trend toward increased CORT levels in animals with a history of adolescent alcohol and subsequent repeated stress, regardless of type. Together, these results suggest that adolescent binge alcohol combined with repeated or chronic stress in adulthood could cause an anxiety-like behavioral phenotype and prolonged HPA axis activation.

Although in this study we observed a decrease in CRF transcript levels in the PVN due to adolescent binge alcohol exposure, our previous studies showed an opposite effect of adolescent binge alcohol on PVN CRF (Przybycien-Szymanska, Mott, Paul, et al., 2011). However, it is important to note the differences in timing of the tissue collection; in the previous study, brains were collected one hour after either water- or alcohol- treatment, whereas in the current study, brains were collected 24 hours after the last experimental manipulation and immediately after behavioral testing. It is possible that this is due either to a delay in the glucocorticoid-mediated negative feedback in the PVN in the alcohol-exposed animals in the current study, or in the previous study, that the control treatment was sufficient to activate the HPA axis resulting in observed higher CRF levels.

The results presented here should be interpreted with some caution. For example, the relatively low sample size and high variability due to the use of an outbred rat strain raises issues
with the generalizability of our findings. It is possible that, with more animals per group, some of our observations which trended toward significance would have reached a statistically significant threshold, or on the other hand could have introduced more variability and abolished the perceived trend. Furthermore, the timing of behavioral testing and tissue collection (24 hours after the last stressor) differed from our prior studies, making direct comparisons to those studies difficult. For example, the observation that CORT levels in the groups which received alcohol during adolescence and a subsequent stressor during adulthood trended toward an increase, particularly compared to their own control group, might suggest that baseline CORT levels are increased in these animals. Alternatively, this could be interpreted as a delay in the resolution of the stress response; perhaps these animals had not completely recovered from the stressor the previous day, while the animals that receive water during adolescence were able to recover more quickly from subsequent stressors. A third possibility is that adolescent alcohol exposure shifts the plasma CORT circadian rhythm; if this were true, however, one would expect to see elevated CORT levels in all the groups with a history of adolescent binge alcohol exposure. Similar arguments could be made for alternative interpretations of the behavioral trends as well.

While some forms of chronic stress, particularly chronic social defeat stress, result in an anxiety-like behavioral phenotype (Tamashiro et al., 2005), most chronic stressors, particularly chronic mild stress, actually results in a depressive-like phenotype (J. Chen et al., 2015; Willner et al., 1992). This depressive phenotype induced by chronic mild stress can be characterized behaviorally by increased anhedonia in the sucrose preference test and increased immobility in the forced swim test; these behavioral changes can be prevented in adrenalectomized rats, suggesting glucocorticoids play a role in the development of the depressive phenotype (J. Chen et al., 2015). Furthermore, chronic mild stress induced alterations in FKBP5 expression and GR function
throughout various stress-related brain regions (including the hypothalamus, hippocampus, and prefrontal cortex) that could be reversed with antidepressant medication, again demonstrating a role of the HPA axis in the development of depression-like symptoms after chronic stress (Guidotti et al., 2013). It would be important going forward, then, to test the effects of adolescent binge alcohol on the development of depressive-like behaviors in response to chronic stressors.

Parvocellular AVP has been implicated more in the stress response to chronic (versus acute) stressors. Elevated AVP levels have been observed after chronic social defeat stress, repeated restraint stress, but these increases have so far been observed within a couple of hours after the stress exposure (Litvin, Murakami, & Pfaff, 2011; Ma et al., 1999). Notably, we did not observe changes in PVN AVP levels, particularly among those with a history of adolescent binge alcohol exposure, but it is possible that PVN AVP levels spiked after stress and resolved before we collected tissue in the current study. A more complete time course of data collection (both behavioral testing and tissue harvesting) would be beneficial in parsing this issue.

In research relevant to this study, increased Dnmt3a in the medial prefrontal cortex after chronic social defeat stress causes an anxiety-like phenotype in mice (Elliott et al., 2016). However, in that study, the authors did not assess hypothalamic Dnmt’s in anxiety-like behaviors. Our lab has recently demonstrated altered DNA methylation patterns in offspring of adolescent alcohol exposed parents (Asimes et al., 2017). It would be interesting to also assess Dnmt function and DNA methylation patterns in the hypothalami of adolescent alcohol-exposed rats, as this could be one molecular mechanism by which adolescent alcohol use causes long-lasting changes in HPA axis function and behavior, and might then alter the responses to chronic stress in adulthood.
Overall, this study provides novel information regarding the effects of adolescent binge alcohol exposure on the behavioral and neuroendocrine responses to chronic stress in adulthood. Of note is that the data suggest that adolescent alcohol may increase the propensity toward an anxiety-like behavioral phenotype and prolonged HPA axis activation after adult chronic stress. These data are in agreement with data from human patients suggesting that a history of teenage binge drinking contributes to an increased risk for mood disorders (McCambridge et al., 2011; Rose et al., 2014; Viner & Taylor, 2007), and highlight the need for a better understanding of the molecular mechanisms underlying adolescent alcohol exposure’s effects, as mood disorders are a major public health issue.
CHAPTER V
MECHANISTIC INSIGHTS INTO THE EFFECTS OF ADOLESCENT BINGE ALCOHOL ON HPA AXIS DYSFUNCTION

Introduction.

Our laboratory and others have demonstrated that adolescent binge alcohol exposure results in long term hyper-reactivity of the HPA axis (Logrip et al., 2013; Przybycien-Szymanska, Mott, Paul, et al., 2011). In the previous chapters, I have presented evidence that strongly suggests HPA axis dysfunction may underlie or contribute to the development of an anxiety-like phenotype, emphasizing the need to understand how alcohol impacts the HPA axis in this way. However, very few studies have focused on the molecular mechanisms by which alcohol produces long term HPA axis dysfunction, a critical gap in the literature that limits our ability to prevent or reverse the effects of adolescent binge alcohol consumption. We demonstrated that adolescent binge alcohol exposure results in increased PVN CRF expression acutely after adulthood psychological stress or subsequent exposure to alcohol (Przybycien-Szymanska, Mott, Paul, et al., 2011), but as demonstrated in chapter IV, this increase in PVN CRF expression does not persist 24 hours after chronic stress or binge alcohol. Therefore, the cause of adolescent binge alcohol-induced HPA axis hyper-reactivity seems to be due to an increase in CRF levels in the PVN which is resistant to feedback inhibition by glucocorticoids.

To address the molecular mechanisms by which this feedback inhibition is lacking, our laboratory has previously performed a series of in vitro experiments using a PVN-derived cell
The isolation and characterization of IVB cells was first described in 2003, where it was demonstrated that these cells retain many of the characteristics of parvocellular hypothalamic PVN neurons (Kasckow et al., 2003). Subsequently, IVB cells have become a widely used cell line to investigate the molecular mechanisms pertaining to PVN control of the HPA axis (Kageyama & Suda, 2009; Sharma et al., 2013). In our studies, although GR mRNA and protein levels remain constant, ChIP assays performed using the IVB cell line revealed that EtOH treatment prevents GR binding to the CRF promoter (Przybycien-Szymanska, Mott, & Pak, 2011). This suggests that after alcohol exposure, either the CRF gene promoter is altered in such a way that it cannot be bound by GR, or that GR itself dysfunctional. Furthermore, this same study demonstrated that a single dose of EtOH as low as 12.5mM increases CRF promoter activity in a luciferase assay in a GR-dependent manner (Przybycien-Szymanska, Mott, & Pak, 2011). Because the luciferase assay utilized an exogenous reporter construct driven by a CRF promoter, this suggests that the deficit lies with GR itself. Therefore, we have focused our current research in understanding the effects of alcohol exposure on GR.

In animal models of prenatal alcohol exposure, GR nuclear localization is affected in a region-specific manner; GR is increased in the nuclei of hippocampal cells but decreased in the nuclei of prefrontal cortex cells (Allan, Goggin, & Caldwell, 2014; Caldwell, Goggin, Tyler, & Allan, 2014). These observations are interesting for two reasons: 1) both regions can modify HPA axis activity, and 2) it suggests that GR nuclear localization may also be affected in the PVN after alcohol exposure, particularly during a period of brain development. Our previous studies demonstrated a decrease in the ability of GR to interact with the CRF promoter; this finding prompts the question of whether GR nuclear translocation may be decreased in the PVN of animals exposed to binge alcohol during adolescence.
Alcohol exposure has also been demonstrated to affect other proteins which are known to interact with GR. For example, FKBP5, which binds GR and prevents nuclear translocation, is acutely increased in the brains of mice exposed to alcohol in a dose-dependent manner (Kerns et al., 2005). Single nucleotide polymorphisms (SNPs) in the FKBP5 gene exist in the human population, and lower FKBP5 gene expression levels are associated with higher levels of alcohol consumption (B. Qiu et al., 2016), suggesting a complicated interplay among alcohol consumption, FKBP5, and GR activity. Another possible mechanism by which alcohol might affect GR involves phosphorylation of the receptor. Both acute and chronic alcohol use can affect MAPK activity (reviewed in Aroor & Shukla, 2004), and various serine residues in the N-terminus of GR can be phosphorylated by MAPKs, and these phosphorylation events can have gene-specific effects on transcriptional activity (W. Chen et al., 2008). Because GR acts in concert with many other proteins including chaperone proteins, adaptor proteins, coactivator and corepressor proteins, etc., it is important to understand which, if any of these protein:protein interactions are affected by adolescent alcohol exposure.

Here, our aim was to examine the effects of alcohol exposure on the glucocorticoid receptor using both in vitro and in vivo models. For the in vivo studies, we utilized the same peri-pubertal Wistar rat model as described in chapters III and IV, but we euthanized the animals for tissue collection one hour after the last dose of alcohol (or water control) to assess GR target gene expression and GR interactions with known binding partners. The in vitro studies allowed us to assess the effects of repeated alcohol treatment on GR subcellular localization (nuclear vs. cytoplasmic) while holding ligand concentration (DEX, a GR agonist) constant. To our knowledge, these experiments are the first of their kind to assess GR function in the context of adolescent binge alcohol exposure as it relates to HPA axis reactivity. These studies are im-
portant for understanding the molecular mechanisms by which adolescent alcohol exposure causes HPA axis dysfunction, which we must understand in order to develop therapeutics for preventing or reversing the effects of teenage binge drinking. I hypothesized that repeated binge alcohol exposure would reduce GR nuclear localization upon ligand binding, due to altered protein:protein interactions, resulting in dysregulated GR target gene expression.

Results.

*Repeated binge-level alcohol exposure subtly reduces GR nuclear localization in a PVN-derived cell line*

Previous studies in our lab using a PVN-derived cell line (IVB) demonstrated that alcohol exposure increases CRF promoter activity in a GR-dependent manner due to the inability of GR to bind the CRF promoter (Przybycien-Szymanska, Mott, & Pak, 2011). However, in these experiments, the levels of GR transcript or protein were unchanged, suggesting that the same amount of GR behaves differently after alcohol exposure. Because GR must translocate from the cytoplasm to the nucleus in order to exert its effects on transcription, I hypothesized that repeated alcohol exposure might reduce the amount of GR in the nucleus. In order to test this, we exposed IVB cells to either 50mM EtOH, 100nM DEX, both, or neither for three consecutive days for two hours at a time, then isolated the cytoplasmic and nuclear protein fractions from the cells immediately following the last dose to immunoblot for GR. DEX treatment significantly increased the proportion of GR in the nuclear fraction as expected, but there was no effect of EtOH treatment (Fig. 11).
Figure 11: Effects of repeated ETOH or DEX on GR nuclear localization. After three days of denoted treatment, IVB cells were lysed and nuclear and cytoplasmic protein fractions were immunoblotted for GR (representative blot shown in (A)). The proportion of nuclear GR was calculated by dividing the relative (to total protein) GR signal protein in the nuclear fraction by the relative GR signal in both cytoplasmic and nuclear fractions for each sample. The summary data are quantified in (B). Data are expressed as mean ± SEM (n=6), and were analyzed by two-way ANOVA, in which p<0.05 was considered significant. There was a significant main effect of DEX treatment on the fraction of nuclear GR.
Adolescent binge alcohol exposure does not alter the GR:HSP90 interaction in the dorsal hippocampus

Our previous studies indicated that the total levels of GR do not change after binge alcohol exposure (Przybycien-Szymanska, Mott, & Pak, 2011); therefore, one possibility is that the dysfunctional GR signaling observed in our in vitro and in vivo models is due to altered protein:protein interactions. Additionally, because region-specific changes in GR localization have been observed in other alcohol models (Allan et al., 2014; Caldwell et al., 2014), we wanted to test this possibility in specific brain regions separately. We focused on the interaction between GR and the chaperone protein HSP90, because HSP90 is known to be a component of the cytoplasmic GR protein complex. In order to test the hypothesis that the interaction between GR and HSP90 would be increased in rats exposed to adolescent binge alcohol, we harvested brain tissue from rats exposed to our peri-pubertal model one hour after the last dose of alcohol, then performed immunoprecipitation experiments to assess this interaction. However, we did not observe a change in the amount of HSP90 in the GR immunoprecipitation samples from the D.Hipp, nor did we observe significant changes in the total levels of GR or HSP90 in this brain region (Fig. 12).
Figure 12: GR-HSP90 interaction in the dorsal hippocampus after adolescent binge alcohol exposure. D.hipp lysates were subject to immunoprecipitation with a GR-specific antibody, then immunoblotted for HSP90 and GR (representative blot shown in (A)). The HSP90 signal relative to total protein in the i.p. lanes was calculated and quantified in (B). The relative GR signal in the input lanes was calculated and quantified in (C). The relative HSP90 signal was calculated and quantified in (D). Data are expressed as mean ± SEM (n=12-16), and were analyzed by t-test.
Adolescent binge alcohol exposure does not alter the GR:HSP90 interaction in the hypothalamus

We next wanted to test whether the interaction between GR and HSP90 is altered in the hypothalamus, as this brain region is obviously more relevant for controlling HPA axis function. Again, I hypothesized that the interaction between GR and HSP90 would be increased in rats exposed to adolescent binge alcohol. In order to test his hypothesis, we used the same tissue harvested from rats exposed to our peri-pubertal model one hour after the last dose of alcohol, then performed immunoprecipitation experiments to assess this interaction. However, we did not observe a change in the amount of HSP90 in the GR immunoprecipitation samples from the hypothalamus, nor did we observe significant changes in the total levels of GR or HSP90 in this brain region (Fig. 13).
Figure 13: GR-HSP90 interaction in the hypothalamus after adolescent binge alcohol exposure. Hypo lysates were subject to immunoprecipitation with a GR-specific antibody, then immunoblotted for HSP90 and GR (representative blot shown in (A)). The HSP90 signal relative to total protein in the i.p. lanes was calculated and quantified in (B). The relative GR signal in the input lanes was calculated and quantified in (C). The relative HSP90 signal was calculated and quantified in (D). Data are expressed as mean ± SEM (n=7), and were analyzed by t-test.
Some, but not all, GR target genes are dysregulated in the hypothalamus after adolescent binge alcohol exposure.

Our previous studies demonstrated that adolescent binge alcohol exposure increased CRF expression (Przybycien-Szymanska et al., 2010), suggesting a decrease in GR function as a transcription factor (as GR is known to repress CRF expression normally). We therefore assessed whether GR function was globally affected, or if this deficit was gene-specific. In order to test this, we designed qPCR primers against two other genes, PER1 (which is important for maintaining circadian rhythms) and FKBP5 (which modulates GR activity), which have both been validated as GR target genes in brain tissue (Mifsud & Reul, 2016). Using RNA isolated from the hypothalami of rats exposed to our peri-pubertal alcohol paradigm, we observed that PER1 mRNA was increased as expected, but there was no change in FKBP5 mRNA levels (Fig. 14). This suggests that GR’s ability to regulate FKBP5, like CRF, is dysfunctional, which might indicate that the genes that are dysregulated by adolescent alcohol are those specific to regulating HPA axis activity, but not other processes.

**Figure 14: Hypothalamic levels of PER1 and FKBP5.** mRNA levels of PER1 (A), and FKBP5 (B), in the hypothalamus was measured by RT-qPCR relative to the control group. Data are expressed as mean ± SEM (n=7), and were analyzed by t-test where p<0.05 was considered significant (*).
Discussion.

The data presented here contribute to our knowledge regarding the way in which GR function is altered after adolescent binge alcohol exposure, thereby providing novel information to the field regarding which GR processes are altered after repeated alcohol exposure. DEX treatment increased GR nuclear localization as expected; however, repeated EtOH treatment did not significantly decrease the proportion of GR in the nuclear protein fraction. There was a trend for EtOH to decrease nuclear localization and it is possible that even a subtle shift in the amount of nuclear GR might have a large impact on overall cellular function; however, there are likely other mechanisms by which alcohol exerts its effects in PVN cells. Furthermore, using our animal model of adolescent binge alcohol exposure, we demonstrated that GR association with HSP90 is not altered in either the dorsal hippocampus or hypothalamus, suggesting that this particular protein:protein interaction is not what mediates the changes in GR function after binge alcohol. Finally, we observed a lack of change in FKBP5 expression in the hypothalamus, but the expected increase in PER1 expression, which combined with our previous findings that rats exposed to adolescent binge alcohol have higher circulating CORT levels and increased CRF expression in the hypothalamus (Przybycien-Szymanska et al., 2010), suggests that repeated adolescent binge alcohol exposure selectively alters GR’s ability to regulate HPA axis-related target genes, but not other GR target genes involved in circadian rhythms, for example. Collectively, these data provide important information regarding the mechanisms by which alcohol affects GR function, while highlighting the need to better understand GR-mediated HPA axis negative feedback in general.

Our finding that repeated alcohol exposure may slightly reduce GR nuclear localization in IVB cells, though subject to the limitations of the use of an immortalized cell line, is generally
consistent with our previous studies which demonstrated a decrease in GR association with the CRF promoter in vitro after alcohol exposure (Przybycien-Szymanska, Mott, & Pak, 2011), and dysfunctional HPA axis negative feedback in our in vivo model of adolescent binge alcohol exposure (Przybycien-Szymanska, Mott, Paul, et al., 2011). Furthermore, others have demonstrated that alcohol exposure during a different developmental time period (gestation) results in region-specific changes in GR subcellular distribution (Allan et al., 2014; Caldwell et al., 2014). This would suggest that GR nuclear translocation may also be deficient in our animal model of adolescent binge alcohol exposure; however, because the samples in the current study were flash frozen after dissection, it was not possible to assess nuclear localization in the same manner in the tissue samples. Future subcellular fractionation and/or immunofluorescent staining studies using tissue from our animal model would be beneficial and would likely reveal altered GR subcellular localization as well.

In the present study, we were able to use brain tissue samples from our animal model of adolescent binge alcohol exposure for a series of GR co-immunoprecipitation experiments. Ultimately, we did not observe a change in the amount of HSP90 interacting with GR in either the dorsal hippocampus or hypothalamus, suggesting that this interaction is not mediating alcohol’s effects on HPA axis negative feedback. We also attempted to assess the interaction of GR with several other known binding partners, including SRC-1, FKBP5, HDAC-1, CREB, and others, but were not able to detect a specific interaction by co-immunoprecipitation (data not shown), despite reports in the literature demonstrating the existence of these interactions in vitro (Sharma et al., 2013). As we have previously reported, we also did not observe significant changes in the levels of GR in either brain region (Przybycien-Szymanska, Mott, & Pak, 2011), nor did we observe significant changes in HSP90 protein levels. This is in opposition to reports that maternal
binge alcohol alters other chaperone protein (including HSP70) expression (Ramadoss, Liao, Chen, & Magness, n.d.), suggesting that in regard to chaperone protein expression, binge alcohol exposure may exert different effects depending upon the developmental timeframe in which that exposure occurs.

Finally, in the current study, we extended our investigation of adolescent binge alcohol’s effects on GR target gene expression. Previously, we demonstrated that, despite higher circulating CORT levels, rats exposed to adolescent binge alcohol express higher levels of CRF (which is normally repressed by liganded GR) in the PVN (Przybycien-Szymanska et al., 2010). Here, we assessed the effects of adolescent binge alcohol on two other validated neuronal GR target genes whose expression is increased by association with ligand-bound GR: PER1, which is involved in regulating circadian rhythms, and FKBP5, which modulates GR activity by binding the receptor and keeping it sequestered in the cytoplasm (Mifsud & Reul, 2016). Interestingly, while PER1 expression increased in the adolescent alcohol exposed rat hippocampi (as expected, since alcohol exposure increases circulating CORT), FKBP5 expression was not different between the two groups. Combined with our previous studies, this raises the interesting possibility that adolescent binge alcohol exposure may selectively alter GR’s ability to regulate HPA axis related genes, but not other GR target genes. These data also support the hypothesis that it is GR itself that is dysfunctional after adolescent binge alcohol exposure, not the CRF promoter, as other GR target genes are also affected. However, the mechanism by which specific GR target genes are dysregulated after adolescent binge alcohol exposure while others are regulated normally is largely unknown; furthermore, future studies should also investigate more broadly which GR target genes are dysregulated after adolescent binge alcohol exposure to gain an appreciation for the gene ontology of those affected by alcohol.
Overall, the data presented here demonstrate that while repeated binge alcohol results in dysfunction of the HPA axis, possibly through altered GR nuclear translocation, it does not reveal which, if any, protein:protein interactions are responsible for this dysfunction. Also, our data combined with our previous studies suggest that alcohol’s effects on GR dysfunction may be specific to certain target genes, which would also suggest alterations in protein:protein interactions within the nucleus specifically. While some of the data presented here represent the absence of significant results, these experiments were some of the first of their kind to be applied to this model of adolescent binge alcohol exposure, and therefore have contributed novel information to the field. These studies highlight the need to not only continue studying the mechanisms by which alcohol affects HPA axis function at a molecular level, but also underscore the paucity of information regarding the mechanisms underlying GR-mediated HPA axis negative feedback in general.
CHAPTER VI

CHARACTERIZATION OF THE GLUCOCORTICOID RECEPTOR INTERACTOME

Introduction.

Glucocorticoids are pleiotropic hormones that act on numerous tissues throughout the body. Normal circulating levels of glucocorticoids follow a circadian rhythm, peaking just before the awake period, and exposure to a physical or psychological stressor causes increased glucocorticoid release via activation of the HPA axis (S. M. Smith & Vale, 2006). Glucocorticoids can bind two different nuclear receptors, the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR); however, most of the stress-induced actions of glucocorticoids are believed to be mediated through GR (Ratka et al., 1989; Reul & de Kloet, 1985). Canonically, when GR is occupied by ligand, it translocates from the cytoplasm to the nucleus of the cell where it exerts transactivation or transrepression of target gene expression (Vandevyver, Dejager, & Libert, 2012). Recent evidence suggests GR also exerts non-genomic effects on the cell which occur with much faster temporal resolution (Vernocchi et al., 2013). Depending on the cell type, GR signaling can modulate metabolic processes, regulate inflammation and immune responses, and control many other functions in peripheral tissues (Chrousos & Gold, 1992). In the brain, glucocorticoids have been shown to increase learning and memory, improve cognition, and importantly, exert negative feedback on the PVN of the hypothalamus to downregulate HPA axis activity after the resolution of a stressful stimulus (McEwen, 2008). Our lab has demonstrated that adolescent binge alcohol exposure disrupts GR-mediated negative feedback in the hypothalamus,
rendering it hypersensitive to stress long after the cessation of alcohol exposure (Przybycien-Szymanska, Mott, & Pak, 2011; Przybycien-Szymanska, Mott, Paul, et al., 2011). However, the molecular mechanisms by which GR exerts negative feedback in PVN parvocellular neurons is understudied, making it difficult to understand how alcohol might impede this normal process.

The glucocorticoid receptor does not act alone to exert its effects on transcription; rather, it must interact with numerous proteins throughout its life cycle to carry out its functions in the cell. Generally, apo-GR is localized primarily in the cytoplasm, in complex with various chaperone and co-chaperone proteins such as hsp90, FKBP5, PP5, and CyP-40 (Cheung & Smith, 2000). Upon ligand binding, GR is shuttled into the nucleus via dynein-mediated cytoskeletal transport and ultimately passes through the nuclear envelope via importins (Elbi et al., 2004; Freedman & Yamamoto, 2004; Silverstein et al., 1999). Inside the nucleus, GR exerts its transcriptional effects by binding coactivator or corepressor proteins, or by interfering with other transcription factor signaling (Petta et al., 2016). While it was originally believed that GR repressed CRF expression by binding an nGRE sequence in the CRF promoter, more recent studies have demonstrated that direct GR-DNA binding is not necessary to downregulate CRF expression (Malkoski & Dorin, 1999; Reichardt et al., 1998). Some have suggested that instead, GR interferes with PKA signaling, while others have shown that GR recruits a repressive complex including MeCP2, Dnmt3b, and HDAC1 (Sharma et al., 2013). Others have provided evidence for more indirect fast repression of CRF release via increased endocannabinoid signaling in the PVN via plasma membrane associated GR (Evanson et al., 2010). Indeed, it is possible that all of these mechanisms may come into play to produce glucocorticoid-mediated negative feedback over a long timescale or in response to different types of stressors.
Here, our aim was to characterize the GR interactome in a neuron-like cell line using the proximity-dependent biotinylation technology BioID. Although others have attempted to describe the GR interactome in some peripheral tissues (Petta et al., 2016), this is the first study to systematically attempt to classify the neuronal GR interactome, which is likely somewhat different from that of peripheral tissues since GR signaling is so cell-type specific. Furthermore, it is the first study to utilize this recent technology to investigate GR protein:protein interactions. Therefore, I hypothesized that this study would identify novel GR interacting proteins that have previously been undetected by traditional techniques in non-neuronal cell types. To test this, we expressed a fusion protein consisting of the human GR tagged on its N-terminus by a promiscuous biotin ligase, BirA*, in the human neuroblastoma derived cell line SK-N-SH. After transient transfection, excess biotin was supplemented in the culture media, and in some circumstances, the GR agonist dexamethasone (DEX) was also added. Then, the biotin-tagged, GR-proximal proteins were purified from cell lysates using streptavidin beads and identified by mass spectrometry. This study identified several new potential GR interacting proteins, but these interactions should be characterized more thoroughly using orthogonal techniques.

Results.

Validation of BioID-GR

The BioID-GR construct, consisting of the human glucocorticoid receptor fused on its N-terminus to a myc-tagged BirA-R188G humanized promiscuous biotin ligase in the pcDNA3.1 backbone, was created by Gibson assembly cloning. Then, the BioID-GR construct or the BioID (ligase only) construct was transfected into HEK293T cells for preliminary validation experiments. To assess construct expression, Western blots using anti-myc and anti-GR primary antibodies revealed a strong myc-immunoreactive band at ~35kD in the BioID lane, and a myc- and
GR-immunoreactive band at ~125kD in the BioID-GR lane, each corresponding to the expected molecular weights of the proteins expressed by these constructs (Fig. 15A). Then, lysates from HEK293T cells transfected with either BioID or BioID-GR and incubated with biotin-supplemented media were subjected to purification by pull-down with streptavidin-coated magnetic beads and resolved by Western blot, to validate proper function of the biotin ligase activity. Staining with an HRP-conjugated streptavidin revealed a diffuse pattern of biotinylated proteins in the BioID transfected samples, while the BioID-GR transfected samples had a more restricted pattern of biotinylated proteins. Importantly, no biotinylated proteins were detected in the “flow through” (i.e. unbound) protein fractions (Fig. 15B top panel). To assess whether known GR-interacting proteins were present after streptavidin bead pull-down, the same membrane was probed with an anti-HDAC-1 antibody, as HDAC-1 has been demonstrated to be part of a repressive GR complex (Sharma et al., 2013). HDAC-1 immunoreactive bands were present in the input and pull-down lanes of both samples (Fig. 15B bottom panel). Next, to assess the functionality of the GR portion of the BioID-GR construct, HEK293T cells transfected with BioID-GR were treated with dexamethasone or vehicle, then subjected to nuclear and cytoplasmic protein fractionation. After resolving the protein fractions via Western blot using an anti-GR primary antibody, the nuclear proportion of GR was calculated. The DEX-treated samples did have a higher proportion of nuclear GR (both endogenous GR and BioID-GR, detected at 90kD and 125kD, respectively) compared to vehicle-treated (Fig. 15C and D).
Figure 15: Validation of BioID-GR function. A) Western blot of HEK293T lysates after transfection with BioID (ligase only, left lane, expected MW: 35kD, bottom arrow) or BioID-GR (fusion protein, right lane, expected MW: 125kD, top arrow). B) Western blot of transfected HEK293T lysates after incubation with biotin-supplemented media. Top panel: staining with streptavidin-HRP; bottom panel: anti-HDAC1 primary antibody. C) Western blot of HEK293T lysates after DEX or vehicle treatment and nuclear/cytoplasmic protein fractionation. Top arrow: BioID-GR; bottom arrow: endogenous GR. D) Quantification of the proportion of nuclear GR staining (normalized to actin) from panel C.
Identification of biotinylated proteins from BioID-GR lysates

To assess the putative GR interactome in a neuron-like cell system, the GR-BioID construct (or the BioID or GFP-GR constructs as controls) was transfected into the human neuroblastoma-derived cell line, SK-N-SH. After transfection, the cells were treated with biotin-supplemented media containing either DEX or vehicle, then lysed after 24 hours of treatment. Each condition (construct/treatment) was performed in biological triplicate. The lysates were subjected to streptavidin bead pull-down to purify the biotinylated proteins, which were then resolved on an SDS-PAGE gel. Each gel lane was cut into five fractions, then prepared for LC-MS/MS by in-gel digestion. Identification by LC-MS/MS was conducted in collaboration with the Midwest Proteome Center at Rosalind Franklin University on a Thermo Orbitrap Elite mass spectrometer equipped with a Dionex Ultimate 3000 RSLC-nano LC. Protein “hits” were identified by PEAKS 8.0 software, with a PEAKS score ≥ 60 and at least two unique peptides corresponding to the identified protein. The hits were further refined by excluding proteins which were inappropriately sized for the fraction in which they were identified. For a hit to be considered associated with either or both of the BioID-GR samples, it had to be unique to at least one of the replicates of that condition, or if it was identified in all three biological replicates of a given condition, it was permitted to have been identified in one of the control samples. Table 3 lists proteins associated with both BioID-GR vehicle and BioID-GR DEX samples. Table 4 lists proteins associated with only the BioID-GR vehicle-treated samples. Table 5 lists proteins associated with only the BioID-GR DEX-treated samples.
<table>
<thead>
<tr>
<th>Protein name (Gene)</th>
<th>Accession</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription activator BRG1 (SMARCA4)</td>
<td>P51532</td>
<td>SMCA4_HUMAN</td>
</tr>
<tr>
<td>Matrilin-2 (MATN2)</td>
<td>O00339</td>
<td>MATN2_HUMAN</td>
</tr>
<tr>
<td>Neuropilin-1 (NRP1)</td>
<td>O14786</td>
<td>NRP1_HUMAN</td>
</tr>
<tr>
<td>Collagen alpha-1(VI) chain (COL6A1)</td>
<td>P12109</td>
<td>CO6A1_HUMAN</td>
</tr>
<tr>
<td>Nuclear autoantigen Sp-100 (SP100)</td>
<td>P23497</td>
<td>SP100_HUMAN</td>
</tr>
<tr>
<td>Glucocorticoid receptor (NR3C1)</td>
<td>P04150</td>
<td>GCR_HUMAN</td>
</tr>
<tr>
<td>SRSF protein kinase 1 (SRPK1)</td>
<td>Q96SB4</td>
<td>SRPK1_HUMAN</td>
</tr>
<tr>
<td>Cell division cycle 5-like protein (CDC5L)</td>
<td>Q99459</td>
<td>CDC5L_HUMAN</td>
</tr>
<tr>
<td>Tubulin beta-2B chain (TUBB2B)</td>
<td>Q9BVA1</td>
<td>TBB2B_HUMAN</td>
</tr>
<tr>
<td>Glutathione S-transferase kappa 1 (GSTK1)</td>
<td>Q9Y2Q3</td>
<td>GSTK1_HUMAN</td>
</tr>
<tr>
<td>Serpin B12 (SERPINB12)</td>
<td>Q96P63</td>
<td>SPB12_HUMAN</td>
</tr>
<tr>
<td>Thioredoxin (TXN)</td>
<td>P10599</td>
<td>THIO_HUMAN</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 3 subunit A (EIF3A)</td>
<td>Q14152</td>
<td>EIF3A_HUMAN</td>
</tr>
<tr>
<td>116 kDa U5 small nuclear ribonucleoprotein component (EF-TUD2)</td>
<td>Q15029</td>
<td>U5S1_HUMAN</td>
</tr>
<tr>
<td>Gem-associated protein 6 (GEMIN6)</td>
<td>Q8WXD5</td>
<td>GEMI6_HUMAN</td>
</tr>
</tbody>
</table>

Table 3: Proteins identified in both BioID-GR samples. Bold face type denotes proteins identified in more than one biological replicate from each treatment condition. Gray text denotes proteins identified in only one vehicle-treated and one dexamethasone-treated BioID-GR sample.
<table>
<thead>
<tr>
<th>Protein name (Gene)</th>
<th>Accession</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-dependent 6-phosphofructokinase platelet type (PFKP)</td>
<td>Q01813</td>
<td>PFKAP_HUMAN</td>
</tr>
<tr>
<td>Leucine-rich repeat-containing protein 17 (LRRC17)</td>
<td>Q8N6Y2</td>
<td>LRC17_HUMAN</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein 6 (HSPA6)</td>
<td>P17066</td>
<td>HSP76_HUMAN</td>
</tr>
<tr>
<td>Gelsolin (GSN)</td>
<td>P06396</td>
<td>GELS_HUMAN</td>
</tr>
<tr>
<td>Plasma membrane calcium-transporting ATPase 4 (ATP2B4)</td>
<td>P23634</td>
<td>AT2B4_HUMAN</td>
</tr>
<tr>
<td>Ephrin type-A receptor 2 (EPHA2)</td>
<td>P29317</td>
<td>EPHA2_HUMAN</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein 1-like (HSPAIL)</td>
<td>P34931</td>
<td>HS71L_HUMAN</td>
</tr>
<tr>
<td>Coatamer subunit beta (COPB2)</td>
<td>P35606</td>
<td>COPB2_HUMAN</td>
</tr>
<tr>
<td>Leucine-rich PPR motif-containing protein mitochondrial (LRPPRC)</td>
<td>P42704</td>
<td>LPPRC_HUMAN</td>
</tr>
<tr>
<td>Protein phosphatase 1G (PPM1G)</td>
<td>O15355</td>
<td>PPM1G_HUMAN</td>
</tr>
<tr>
<td>Complement component C9 (C9)</td>
<td>P02748</td>
<td>CO9_HUMAN</td>
</tr>
<tr>
<td>Merlin (NF2)</td>
<td>P35240</td>
<td>MERL_HUMAN</td>
</tr>
<tr>
<td>Importin subunit beta-1 (KPNB1)</td>
<td>Q14974</td>
<td>KPNB1_HUMAN</td>
</tr>
<tr>
<td>Pumilio domain-containing protein KIAA0020 (KIAA0020)</td>
<td>Q15397</td>
<td>KIAA0020_HUMAN</td>
</tr>
<tr>
<td>Zinc finger protein 800 (ZNF800)</td>
<td>Q2TB10</td>
<td>ZNF800_HUMAN</td>
</tr>
<tr>
<td>Sp110 nuclear body protein (SP110)</td>
<td>Q9HB58</td>
<td>SP110_HUMAN</td>
</tr>
<tr>
<td>Protein SDA1 homolog (SDAD1)</td>
<td>Q9NVU7</td>
<td>SDA1_HUMAN</td>
</tr>
<tr>
<td>Adenosylhomocysteinase (AHCY)</td>
<td>P23526</td>
<td>SAHH_HUMAN</td>
</tr>
<tr>
<td>Elongation factor 1-gamma (EEF1G)</td>
<td>P26641</td>
<td>EEF1G_HUMAN</td>
</tr>
<tr>
<td>Signal recognition particle 19 kDa protein (SRP19)</td>
<td>P09132</td>
<td>SRP19_HUMAN</td>
</tr>
<tr>
<td>Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1 (RPN1)</td>
<td>P04843</td>
<td>RPN1_HUMAN</td>
</tr>
</tbody>
</table>

Table 4: Proteins identified in only BioID-GR vehicle samples. Bold face type denotes proteins identified in more than one biological replicate. Gray text denotes proteins identified in only one replicate.
<table>
<thead>
<tr>
<th>Protein name (Gene)</th>
<th>Accession</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-rich interactive domain-containing protein 1B (ARID1B)</td>
<td>Q8NFD5</td>
<td>ARI1B_HUMAN</td>
</tr>
<tr>
<td>SWI/SNF complex subunit SMARCC2 (SMARCC2)</td>
<td>Q8TAQ2</td>
<td>SMRC2_HUMAN</td>
</tr>
<tr>
<td>Nuclear receptor coactivator 2 (NCOA2)</td>
<td>Q15596</td>
<td>NCOA2_HUMAN</td>
</tr>
<tr>
<td>Mediator of RNA polymerase II transcription subunit 1 (MED1)</td>
<td>Q15648</td>
<td>MED1_HUMAN</td>
</tr>
<tr>
<td>TOX high mobility group box family member 4 (TOX4)</td>
<td>O94842</td>
<td>TOX4_HUMAN</td>
</tr>
<tr>
<td>Zinc finger and BTB domain-containing protein 11 (ZBTB11)</td>
<td>O95625</td>
<td>ZBTB11_HUMAN</td>
</tr>
<tr>
<td>RNA-binding protein 25 (RBM25)</td>
<td>P49756</td>
<td>RBM25_HUMAN</td>
</tr>
<tr>
<td>Serine/threonine-protein kinase TAO1 (TAOK1)</td>
<td>Q7L7X3</td>
<td>TAOK1_HUMAN</td>
</tr>
<tr>
<td>Zinc finger protein 512B (ZNF512B)</td>
<td>Q9KMK6</td>
<td>ZN512B_HUMAN</td>
</tr>
<tr>
<td>Putative helicase MOV-10 (MOV10)</td>
<td>Q9HCE1</td>
<td>MOV10_HUMAN</td>
</tr>
<tr>
<td>Yorkie homolog (YAP1)</td>
<td>P46937</td>
<td>YAP1_HUMAN</td>
</tr>
<tr>
<td>Myocyte-specific enhancer factor 2D (MEF2D)</td>
<td>Q14814</td>
<td>MEF2D_HUMAN</td>
</tr>
<tr>
<td>Coagulation factor X (F10)</td>
<td>P00742</td>
<td>FA10_HUMAN</td>
</tr>
<tr>
<td>28S ribosomal protein S5 mitochondrial (MRPS5)</td>
<td>P82675</td>
<td>RT05_HUMAN</td>
</tr>
<tr>
<td>Histone H1x (H1FX)</td>
<td>Q92522</td>
<td>H1X_HUMAN</td>
</tr>
<tr>
<td>Glutathione peroxidase 1 (GPX1)</td>
<td>P07203</td>
<td>GPX1_HUMAN</td>
</tr>
<tr>
<td>Peptidyl-prolyl cis-trans isomerase B (PPIB)</td>
<td>P23284</td>
<td>PPIB_HUMAN</td>
</tr>
<tr>
<td>Serpin B4 (SERPINB4)</td>
<td>P48594</td>
<td>SPB4_HUMAN</td>
</tr>
<tr>
<td>Hemoglobin subunit beta (HBB)</td>
<td>P68871</td>
<td>HBB_HUMAN</td>
</tr>
<tr>
<td>Calmodulin-like protein 5 (CALML5)</td>
<td>Q9NZT1</td>
<td>CALL5_HUMAN</td>
</tr>
<tr>
<td>Laminin subunit alpha-5 (LAMA5)</td>
<td>O15230</td>
<td>LAMA5_HUMAN</td>
</tr>
<tr>
<td>Growth arrest and DNA damage-inducible proteins-interacting protein 1 (GADD45GIP1)</td>
<td>Q8TAE8</td>
<td>G45IP_HUMAN</td>
</tr>
<tr>
<td>SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3 (SMARCD3)</td>
<td>Q6ST5</td>
<td>SMRD3_HUMAN</td>
</tr>
</tbody>
</table>

**Table 5: Proteins identified in only BioID-GR dexamethasone samples.** Bold face type denotes proteins identified in more than one biological replicate. Gray text denotes proteins identified in only one replicate.
Proteins identified by BioID-GR are enriched in nucleic acid binding proteins and proteins with known interactions

After the protein hit refinement described above, the lists of protein hits were pooled and subject to further analysis. PANTHER protein class analysis (pantherdb.org) revealed enrichment of nucleic acid binding proteins and transcription factors in both the high-confidence associated protein list (the 16 bold face type proteins listed in Tables 3-5) and the total associated protein lists (all 59 proteins listed in Table 3-5) (see Fig. 16). Furthermore, STRING analysis (string-db.org) revealed that the combined dataset (consisting of all 59 proteins identified in the BioID-GR samples) was significantly enriched in interacting proteins (PPI enrichment p-value = 0.00111), though this does not necessarily mean the dataset is enriched for physically interacting proteins, just biological interactions (see Fig. 17). Notably, only two of the 16 high-confidence associated proteins (BRG1/SMARCA4 and SMARCC2) have been previously reported to interact with GR directly in other organisms, though others (i.e. ARID1B) have been demonstrated to interact with GR-interacting proteins. Furthermore, other proteins known to interact with GR (i.e. NCOA2) were identified in the total set of 59 associated proteins, while other known GR interacting proteins, such as HSP90, were identified in the BioID-GR samples as well as other control samples (BioID or GFP-GR transfected samples), and therefore excluded from these lists.
Figure 16: PANTHER protein class analysis of BioID-GR associated proteins. A) PANTHER analysis of the 16 high-confidence BioID-GR associated proteins. B) PANTHER analysis of all 59 BioID-GR associated proteins. White numbers indicate the number of proteins in the designated protein class.
Figure 17: STRING analysis of BioID-GR associated proteins. STRING analysis depicting the types of biological interactions known between the 59 BioID-GR associated proteins identified.
Discussion.

Here, we have identified a cohort of proteins which are proximal to (and potentially interact with) the glucocorticoid receptor in a neuron-like cell line using the proximity dependent biotinylation technology, BioID. This includes a total of 59 proteins, 16 of which are high-confidence protein hits, the vast majority of which are novel putative GR-interacting proteins. As one might have expected, transcription factors and other nucleic acid binding proteins are enriched in the lists of putative GR-interacting proteins. These data contribute novel information regarding the neuronal GR interactome, and lay the framework for further studies to validate and further investigate these new interactions in normal GR signaling and in the development of disease.

Some of the proteins identified by the BioID-GR screen were already known to be GR-interacting proteins, lending validity to the screen itself. Importantly, as GR is known to homodimerize, GR itself was identified in all of the BioID-GR transfected samples (and none of the control samples). Another example, the transcription activator BRG1 (SMARCA4), here identified in both BioID-GR conditions, has been previously characterized to interact with GR and other nuclear receptor family members as part of larger chromatin remodeling complexes, including the neural progenitors-specific chromatin remodeling complex (npBAF complex) and the neuron-specific chromatin remodeling complex (nBAF complex) (Archer & Fryer, 1998). In the mammalian system, this has been shown to be an indirect interaction mediated by BRG1-associated factor 60a (BAF60a), and GR mutants that cannot bind BAF60a and therefore cannot bind the larger BRG1 complex cannot properly activate transcription via chromatin remodeling (Hsiao, Fryer, Trotter, Wang, & Archer, 2003). Additionally, in the DEX treated BioID-GR samples, we identified two other components of the nBAF/npBAF complexes: AT-rich interac-
tive domain-containing protein 1B (ARID1B) and SWI/SNF complex subunit SMARCC2 (SMARCC2) (Hurlstone, Olave, Barker, van Noort, & Clevers, 2002; Kadam et al., 2000), lending further support that the observation of these proteins in our screen indeed represents a true interaction between GR and these three “hits.” In neuronal cells specifically, BRG1 has also been demonstrated to form a repressive complex with Rb and HDAC-1 at the c-Fos promoter, which is then undergoes a “switch” during neuronal stimulation and calcium influx, allowing for activity-dependent c-Fos transcription (Z. Qiu & Ghosh, 2008). However, this particular model has not thus far incorporated nuclear receptor signaling into the repressive actions of BRG-1.

Some other proteins identified here are related to proteins known to bind GR, or are part of a larger complex that has been demonstrated to be important for GR signaling. For example, we identified tubulin beta-2B chain (TUBB2B) in both BioID-GR transfected conditions. It is now accepted that GR nuclear translocation is mediated by active transport along microtubules, which are composed of tubulins (Galigniana et al., 1998). We additionally identified several other tubulin components throughout both the BioID-GR and control samples, but the beta-2B chain was the only one identified specifically in the BioID-GR samples. In the vehicle-treated BioID-GR samples, we identified heat shock 70 kDa protein 6 (HSPA6), and to a lesser degree of confidence, heat shock 70 kDa protein 1-like (HSPA1L). Hsp70 is well characterized to interact with GR in the cytoplasm, particularly with nascent GR to aid in proper folding/assembly (Pratt, Morishima, Murphy, & Harrell, 2006). Similar to the tubulins identified, we identified hsp70 alpha and beta in both the BioID-GR and other control samples, but these two hsp70 family members were specifically identified in the BioID-GR samples. Collectively, these data support the validity of this screen for the identification of GR-interacting proteins.
The novel, putative GR-interacting proteins identified here must be further validated to confirm a true interaction, but the techniques that may be used to do this (i.e. co-immunoprecipitation, etc.) are much lower throughput than the present screen. Therefore it is important to identify the most interesting or logical candidates that might be a true GR-interacting partner. A handful of the high-confidence BioID-GR associated proteins identified here are of particular interest due to their nuclear localization and activity in modulating transcription and/or splicing. For example, the nuclear autoantigen Sp-100 (SP100) was identified in both BioID-GR conditions. Sp-100 has been demonstrated to act as both a co-activator and co-repressor in conjunction with ETS-1 and ETS-2 (Wasylyk, Schlumberger, Criqui-Filipe, & Wasylyk, 2002; Yordy et al., 2004). Of note is the fact that GR has been demonstrated to interact with ETS-2 in 3T3 murine fibroblast cells (Mullick et al., 2001). Therefore, while this may prove to be an indirect interaction via ETS-1 or -2, the association of GR with Sp-100 should be experimentally validated. Other identified proteins of interest include cell division cycle 5-like protein (CDC5L), which is best known for its role in mediating splicing, but may also act as a transcription activator (Ajuh et al., 2000). SRSF protein kinase 1 (SRPK1) is another interesting putative GR-interacting protein, as it has been shown to regulate numerous cellular processes including splicing, and interacts directly with hsp40, which results in dynamic interactions with hsp70 and hsp90, all chaperones known to also interact with GR (Sanidas et al., 2010; Zhong, Ding, Adams, Ghosh, & Fu, 2009). The other high-confidence BioID-GR associated proteins represent additional interesting potential GR-interacting proteins, but precedence should be given to the proteins listed above due to their logical incorporation into known GR signaling pathways.

There are some limitations to the interpretation of the results of the current study due to the experimental design. First, because we did not use a labeling technique (such as SILAC or
iTRAQ), it was not possible to reliably quantify proteins among different samples, meaning we had to take an all-or-nothing approach to the data analysis. This approach likely led to the exclusion of GR-interacting proteins from the identified “hits,” as highly abundant proteins were likely tagged non-specifically by the ligase-only controls or non-specifically bound to the streptavidin beads. For example, HSP90 (a well-characterized GR-interacting protein) was detected in all samples. Use of a labeling technique may have revealed enrichment of HSP90 and other abundant proteins in the BioID-GR samples, but in the current study, it was not possible to reliably make that distinction. In an attempt to overcome this limitation, we used spectral counting to quantify among the different samples. While a handful of proteins were found to be enriched in the BioID-GR samples, because we could not reliably quantify the total protein amount after streptavidin bead pull-down due to the presence of contaminating streptavidin, this enrichment is likely an artifact. Another major limitation of this study is the inability to distinguish between GR-interacting and GR-proximal proteins. While one might assume that a protein within ~10nm of a protein for a period of time long enough to be labeled by the BirA* enzyme is interacting with the protein of interest (either directly or as part of a macromolecular complex), this assumption is not always true. Therefore, some of the detected hits could be false-positives (i.e., they were in close proximity to GR but not physically interacting with GR), particularly those which were only detected in one of the three biological replicates. Proteins which were detected across multiple biological replicates of the same treatment are more likely to be true interacting partners, but in any case, the identified novel interactions should be validated using orthogonal techniques like co-immunoprecipitation, preferably in conjunction with knock-down experiments. Inevitably, another possible source of error in this screen could be due to the necessity to exogenously over-express the BioID-GR bait protein, and the use of an immortalized cell line which cannot com-
pletely replicate a “natural” cellular environment. While these limitations could potentially be overcome by using alternative techniques (for example, by knocking-in the bait protein using gene editing in primary cultured cells), those methods would be much more labor intensive and would still require further validation due to the other limitations of this type of screen. Finally, a limitation of any proteomic experiment is related to the coverage of the proteome in general. While our study identified a number of proteins from each sample that is in the “ball-park” of other similar proteomic experiments, it is possible that true interactions were not detected because they did not produce tryptic peptides capable of being efficiently detected by LC-MS/MS, or because they were present at such low concentrations compared to other proteins that they were not selected for dissociation and identification by MS2. Some methods to increase this coverage might include using a different enzyme or enzymes to digest the proteins, further fractionating the gel samples, or altering the LC run time or gradient to better separate the peptides. However, the methods used here were standard to the field in general, so while it may not be possible to identify every GR-interacting protein in the cell, we were able to identify several novel putative GR-interacting proteins, thereby moving the field forward.

The full characterization of the GR interactome is a daunting task which cannot be fully accomplished by one study. While these experiments lay the framework for a more detailed description of the neuronal GR interactome, there are some important considerations going forward which will aid in this endeavor. First, this study highlights the need to use a labeling technique to quantify among samples to ensure that true GR-interacting proteins are not excluded due to possible non-specific interaction with the ligase-only control (a commonly used control in the field). The ideal method for this would likely be SILAC, in which cells are cultured in heavy- or light-labeled amino acid-supplemented media, such that proteins are labeled prior to lysis and pooled
before streptavidin bead pull-down. This eliminates the need to quantify the proteins after the pull-down, and allows all the samples being compared to be run on the LC-MS/MS at the same time, saving time and eliminating inter-run variability. The disadvantage to this technique, however, is that it limits the number of samples being compared to two or three (if a “medium” labeled culture condition is included). Therefore, one could compare BioID-GR samples treated with DEX or vehicle to one control (untransfected? GFP-GR? Ligase only? Treated or untreated with agonist?), but the choice of that control introduces limitations to the interpretation of the results. An alternative quantification technique would be labeling with iTRAQ or TMT isobaric tags, which can compare quantities of proteins in up to 10 different samples at once. However, this technique requires that proteins be quantified after streptavidin bead pull-down, and would likely require further purification after elution from the beads to suspend the proteins in a buffer compatible with isobaric label tagging. In our hands, we were unable to reliably quantify the proteins after the pull-down due to the presence of contaminating streptavidin that had leached off the beads which overwhelmed the quantity of protein from our actual samples. Another consideration which would generally increase the yield of biotinylated proteins from the same amount of cells would be to create a stable cell line expressing the BioID or BioID-GR constructs. After selecting for transduced cells, this would ensure that ~100% of the cells express the appropriate construct (as opposed to ~30-50% after transient transfection in the SK-N-SH cell line), therefore increasing the proportion of the sample with biotinylated proteins. Furthermore, this would allow for the use of different cell lines that are difficult to transfect but might be sufficiently transduced by retrovirus to create a stable cell line. Low transfection efficiency is the main reason we did not use the PVN-derived IVB cell line for these experiments, but also because IVB cells are derived from rat PVN and our BioID-GR construct expresses the human GR. While it would take
some time to clone the BioID-GR construct into a lentiviral backbone, generate the virus, and select for transduced cells, the investment of a couple months of work would greatly increase the efficiency of the experiment. Finally, as discussed above, future experiments should focus on validating the “hits” identified here (and/or by future iterations of this experiment) using orthogonal techniques.

Overall, the current study has preliminarily identified 59 putative GR-interacting proteins, many of which have not been previously described in the context of GR signaling. Sixteen of these proteins were high-confidence hits (two specific to DEX-treated BioID-GR samples, three specific to vehicle-treated BioID-GR samples, and 11 specific to both BioID-GR conditions), therefore, this short list of proteins should be the first to be validated in follow-up studies. The identified proteins were enriched in transcription factors and nucleic acid binding proteins, which is logical given the glucocorticoid receptor’s known function as a ligand-activated transcription factor. This study provides novel information regarding GR signaling under “normal” conditions; future studies can therefore assess the effects of repeated binge drinking or other chronic stressors on these novel interactions.
CHAPTER VII
FINAL DISCUSSION

Summary.

The mechanisms by which adolescent binge alcohol exposure ultimately leads to HPA axis dysfunction and an increased propensity toward mental health disorders remain unknown. Furthermore, the exact molecular processes by which glucocorticoids exert feedback inhibition on the hypothalamus remains understudied, with various models suggesting both genomic and nongenomic effects mediated by the glucocorticoid receptor. Therefore, the goals of this project were to characterize the behavioral and neuroendocrine responses to various adulthood stressors in our rat model of adolescent binge alcohol exposure, to understand what molecular pathways were disrupted in the adolescent brain after repeated binge alcohol treatment, and to investigate the neural glucocorticoid receptor interactome. *The overarching hypothesis of this work was that binge alcohol exposure during adolescence alters the GR’s interactions with other proteins, resulting in increased CRH expression and aberrant HPA axis signaling, which contributes to inappropriate neuroendocrine and behavioral responses to psychological stress during adulthood.*

In Chapter III, the data demonstrate that rats with a history of adolescent binge alcohol exposure exhibit increased anxiety-like behaviors and a hypersensitive neuroendocrine response after acute psychological stress. In Chapter IV, the data suggest that both homotypic and heterotypic adult stresses cause similarly dysfunctional behavioral and HPA axis changes in rats with a history of adolescent binge alcohol exposure. In Chapter V, the data demonstrate that these changes
due to adolescent binge alcohol exposure are likely not due to significantly altered GR subcellular localization, nor are they due to increased interactions between GR and the chaperone HSP90, suggesting adolescent binge alcohol exposure selectively alters certain GR mediated signaling pathways but not global GR function. In Chapter VI, the data describe known and novel GR interacting proteins identified by a proximity-dependent biotinylation proteomic screen in a neuronal-like cell type. Collectively, these studies provide novel information regarding the molecular mechanisms underlying glucocorticoid receptor mediated HPA axis negative feedback, which is disrupted in response to adolescent binge alcohol exposure, contributing to an increase in mood disorder susceptibility.
Figure 18: Graphical summary of key findings. Adolescent binge alcohol exposure combined with acute or chronic stress in adulthood increases anxiety-like behavior and HPA axis sensitivity. Inlay: Schematic of CRF neuron GR signaling, denoting putative novel GR-interacting proteins identified by BioID-GR.
Key Findings

Chapter III: Effects of adolescent binge alcohol on behavioral and neuroendocrine responses to acute psychological stress

- History of adolescent binge alcohol exposure and acute restraint stress increased risk assessment behaviors
- History of binge alcohol exposure alone did not increase canonical anxiety-like behaviors or alter ethological behaviors
- Both history of binge alcohol exposure and acute restraint stress increased plasma CORT
- Acute restraint increased cFos and CRF expression, decreased GR in V.Hipp

Chapter IV: Effects of adolescent binge alcohol on behavioral and neuroendocrine responses to chronic stressors

- Animals with a history of adolescent binge alcohol exposure trended toward an anxiety-like phenotype in response to a chronic adulthood stressor
- Repeated homotypic (binge alcohol) and heterotypic (restraint) stressors had similar effects in animals previously exposed to binge alcohol during adolescence
- History of adolescent binge alcohol exposure decreased PVN CRF expression after behavioral testing in all groups
- Animals with a history of adolescent binge alcohol exposure may have a prolonged CORT response to chronic stressors
Key Findings

Chapter V: Glucocorticoid receptor function after repeated adolescent binge alcohol exposure

- Adolescent binge alcohol exposure did not alter GR-HSP90 interactions or expression of either protein in the dorsal hippocampus or whole hypothalamus.
- Adolescent binge alcohol exposure increased PER1 expression in the hypothalamus, but not hypothalamic FKBP5 expression (both are neuronal GR target genes).
- Repeated 50mM EtOH exposure did not significantly decrease DEX-induced GR nuclear localization in IVB cells.

Chapter VI: Characterization of the glucocorticoid receptor interactome

- The BioID-GR construct used in these experiments was validated to be functional.
- 59 putative GR interacting proteins were identified by the BioID screen, including 16 high-confidence hits, the majority of which had not been previously reported to interact with GR.
- The identified proteins were enriched for transcription factors and nucleic acid binding proteins.
Final Thoughts.

Adolescent binge alcohol exposure and mood disorder development

The data presented in Chapters III and IV support the hypothesis that adolescent binge alcohol exposure promotes the development of an anxiety-like phenotype, specifically in combination with exposure to an acute or chronic stressor during adulthood. This is in agreement with numerous other animal studies that suggest adolescent alcohol exposure not only causes an anxiety-like phenotype, but also depression-like phenotypes. For example, i.p. alcohol injections during adolescence increase adulthood anxiety-like behaviors in the light-dark box and elevated-plus maze tests (Pandey et al., 2015). This anxiety-like phenotype is not solely based on behavioral observations; rodent studies have also demonstrated that adolescent binge alcohol exposure results in hypersensitivity of the HPA axis (Brunell & Spear, 2005; Przybycien-Szymanska, Mott, Paul, et al., 2011). Furthermore, adolescent alcohol exposure has been demonstrated to result in anhedonia, a depressive-like behavior, in multiple animal models (Briones & Woods, 2013b; Varlinskaya, Kim, & Spear, 2017). It is important to acknowledge that not all animal studies that have investigated mood disorder-like behaviors after adolescent binge alcohol exposure have observed statistically significant differences (Coleman et al., 2011); however, the collective body of evidence does point to a causative role of adolescent binge alcohol exposure in the development of these disorders.

These animal studies are also in agreement with human epidemiological studies which demonstrate that teenage binge drinking increases the risk of developing a mood disorder later in life (McCamine et al., 2011; Rose et al., 2014; Viner & Taylor, 2007). Furthermore, HPA axis dysfunction in adolescent binge drinkers has been observed, lending further support to the idea that the development of mood disorders after adolescent binge alcohol use might be mediated by
changes to HPA axis reactivity (Wemm et al., 2013). This is important, not only because of the great socioeconomic burden that mood disorders place on our society, but also because both alcohol consumption and mood disorders can alter other cognitive aspects, including learning and memory tasks (Goldstein, Déry, Pilgrim, Ioan, & Becker, 2016).

Adolescent binge alcohol exposure and sex differences

In the current studies, the effects of adolescent binge alcohol exposure on the adult responses to acute and chronic stress were only assessed in male rats. This is because previous studies from our laboratory demonstrated that the effects of adolescent binge alcohol on the HPA axis are sexually dimorphic; female rats do not exhibit the same alterations in CRF or AVP gene expression as observed in males, and their CORT response does habituate to repeated alcohol doses (Przybycien-Szymanska et al., 2010). Furthermore, we demonstrated that these sex differences were due to the presence of 17β-estradiol, as ovariectomized adolescent rats do not habituate to repeated alcohol doses (Przybycien-Szymanska et al., 2012). Interestingly, studies using Sprague-Dawley rats administered alcohol vapors during adolescence that were subsequently challenged with intragastric alcohol during adulthood demonstrated that male rats exhibited an increase in PVN CRF mRNA in response to adult alcohol challenge, and this CRF increase was abolished by a history of adolescent alcohol, while female rats exhibited an increase in PVN AVP mRNA that was blunted by a history of adolescent alcohol exposure (Logrip et al., 2013). This suggests that the HPA axis is differentially affected by alcohol in males and females during adulthood and in the context of adolescent alcohol exposure. Therefore, future studies using our laboratory’s animal model could continue to investigate the effects of adolescent binge alcohol exposure on adult behavioral and neuroendocrine outcomes, but experiments using female rats would likely produce different results than those that were obtained in the current studies.
Human studies have also demonstrated that males and females can exhibit deleterious effects of teen binge drinking, further emphasizing the need to characterize these sex differences in animal models. For example, female college students with self-reported problematic drinking habits exhibited dysfunctional physiologic stress responses (measured by salivary cortisol levels) in response to a psychological stress; however, males were not assessed in this study (Wemm et al., 2013). Imaging studies have also demonstrated sexually dimorphic effects of binge drinking: female binge drinkers have increased brain volume in areas relevant to inhibitory control and motivated behaviors compared to their healthy counterparts, whereas male binge drinkers exhibited decreases in volume in these areas compared to healthy males (Kvamme et al., 2016). However, it is difficult to determine if these sexually dimorphic changes are an effect of binge drinking, or if they had existed prior to the onset of alcohol consumption. Longitudinal studies have failed to find sex differences in the developmental changes in brain region volumes due to the onset of binge drinking, suggesting any sex differences may have existed prior to binge drinking onset (Squeglia et al., 2015).

Whether or not the effects of adolescent alcohol use produce sexually dimorphic effects on the individuals who themselves choose to engage in binge drinking, ongoing research in our laboratory and others aims to investigate the effects of adolescent binge alcohol exposure on the outcomes of offspring with no direct exposure to adolescent alcohol themselves. We observed numerous differentially methylated cytosine residues (DMCs) in the hypothalami of male rat pups born to parents with a history of adolescent alcohol exposure; furthermore, these DMCs were different depending on if the “family” history of adolescent binge drinking came only from the maternal side, the paternal side, or from both parents (Asimes et al., 2017). In human studies, children from a family with multiple alcohol-dependent relatives have an increased risk of be-
coming alcohol dependent themselves (Hill, Tessner, & McDermott, 2011). Indeed, environmental factors likely play an overwhelming role in these families, but a better understanding of the biological mechanisms by which alcohol’s effects can be passed between (male or female) parent and offspring are necessary to be able to effectively manage these intergenerational changes.

*Alcohol and other stressors*

The results of Chapter III demonstrate that adolescent binge alcohol exposure leads to an anxiety-like phenotype (behaviorally and in terms of HPA axis function) in response to an acute psychological stressor during adulthood. However, while we observed increases in risk assessment, which is likely a component in the development of an anxiety disorder, we did not observe increases in the amount of time spent in the closed arms of the EPM or a decrease in the time spent in the open arms. This is in contrast to some other studies in the field, which utilized slightly different models of adolescent binge alcohol exposure (Briones & Woods, 2013b; Pandey et al., 2015). Although an acute stressor is normally expected to activate the HPA axis over a short period of time, it should not result in long term behavioral or neuroendocrine dysfunction. However, this is exactly the case in patients with PTSD, in which a single traumatic event results in long term behavioral and neuroendocrine symptoms (Carrasco & Van de Kar, 2003; Simeon et al., 2007). Because of the timing of the current study, it is unclear if the increases in anxiety-like behavior that we observed in animals with a history of adolescent binge alcohol exposure and adult acute stress would persist for hours, days, or longer. If these differences were to persist, this then raises questions regarding the role of teenage binge drinking in relation to subsequent adult activities. For example, due to the likelihood that an individual might encounter a traumatic, near-death event in military combat, might screening for a history of teen binge drinking be included in military intake questionnaires? Should these individuals be prevented from holding
combat positions in the military, or should they continue to do the same work but be monitored more closely? As our understanding of the effects of teenage binge drinking unfolds, these societal questions will undoubtedly need to be addressed as well.

The results of Chapter IV demonstrate that adolescent binge alcohol exposure increases the propensity toward an anxiety-like phenotype in rats subsequently exposed to some sort of repeated stress during adulthood, whether that is a homotypic (alcohol) or heterotypic (restraint) stressor. This supports the general idea that adolescent binge alcohol consumption can lead to the development of mood disorders, particularly in addition to other chronic stressors later in life. Others have demonstrated that adolescent binge alcohol exposure combined with repeated adulthood stress likewise results in social deficits and a depressive-phenotype (Varlinskaya et al., 2017). Although that particular study used a different rat model of adolescent binge alcohol (Sprague-Dawley rats administered alcohol from PND25-45 every other day), they also used repeated restraint stress as the adulthood stressor, as we did for one of the groups in our study. The observation that adolescent binge alcohol also results in increased depressive behaviors after adult chronic stress is extremely important, as chronic stress itself is known to be a stimulus for the development of depression (J. Chen et al., 2015; Willner et al., 1992). Furthermore, we observed dysfunctional changes to HPA axis effector levels, and HPA axis dysfunction is also known to be associated with depression, particularly after chronic stress (Guidotti et al., 2013; Naughton et al., 2014). Collectively, this suggests that adolescent binge alcohol exposure alters the resulting responses to adulthood chronic stressors in such a way that furthers the development of mood disorders.

It is important to note that while these studies have investigated the effects of acute and chronic systemic or psychological stressors in the context of adolescent binge alcohol exposure,
that alcohol itself not only activates the systemic neuroendocrine stress response, but also produces cellular stress, primarily in the form of oxidative stress. In the brain, this oxidative stress has been associated with mitochondrial dysfunction, long term decreases in synaptic plasticity, neuroinflammation, increases in cell death, and ultimately behavioral deficits in learning and memory paradigms (Hansson et al., 2010; Tajuddin, Przybycien-Szymanska, Pak, Neafsey, & Collins, 2013; Tapia-Rojas et al., 2017). Interestingly, inhibition of fatty acid amide hydrolase (FAAH) and the resulting increase in anandamide has been demonstrated to reduce oxidative stress in the prefrontal cortex of adolescent rats (Pelição et al., 2016). Whether oxidative stress plays a role specifically in the dysfunction of glucocorticoid feedback inhibition in the PVN remains to be seen, but similar therapeutics targeting antioxidant pathways might be useful if that is proven to be the case.

*The effects of alcohol on glucocorticoid signaling*

Previous studies from our lab using the rat PVN-derived cell line, IVB, have demonstrated that alcohol treatment increases CRF promoter activity and decreases GR binding to the CRF promoter region (Przybycien-Szymanska, Mott, & Pak, 2011). The results of chapter V suggest that this CRF promoter dysregulation is not due to major changes in GR subcellular localization. While these observations are helpful in deciphering potential molecular pathways affected by alcohol exposure, due to the general limitations of using an immortalized cell line (absence of feedback from other cell types, inability to replicate the adolescent developmental period, etc.), these data alone are insufficient to draw meaningful conclusions based on the molecular effects of adolescent binge alcohol exposure in an entire organism. Therefore, Chapter V also investigated the functionality of GR in our *in vivo* adolescent binge alcohol model, and demonstrated that only some GR target genes seem to be specifically affected by alcohol, and that global GR
binding to the chaperone HSP90 is not affected in the hippocampus or hypothalamus. Although it was not possible to assess GR nuclear localization in samples from our animal model, collectively the in vitro data and the qPCR data from the animal tissue samples suggest that changes in GR nuclear localization are NOT the major cause of adolescent alcohol-induced dysfunction of CRF expression and HPA axis negative feedback.

Unfortunately, there are few other studies that have investigated the effects of alcohol on GR, and even fewer that specifically investigated the role of adolescent binge alcohol exposure (as opposed to fetal or more chronic abuse models) on GR function. Animal models of chronic alcohol exposure have demonstrated decreases in GR expression in various brain regions including during alcohol withdrawal; furthermore, alcohol withdrawal also resulted in decreased GR binding to a GRE probe (Roy, Mittal, Zhang, & Pandey, 2002). It is important to note, however, that our animal model of adolescent binge alcohol exposure does NOT produce traditional alcohol withdrawal effects (i.e. seizure activity) (Callaci et al., 2004; Przybycien-Szymanska et al., 2010), so it is unclear how these data might translate to subclinical drinking patterns in human adolescents. On another note, in models of fetal alcohol exposure, region-specific changes in GR subcellular localization have been observed (Allan et al., 2014; Caldwell et al., 2014). These data are in contrast with the data presented in Chapter V, though this discrepancy is likely due to the administration of alcohol during completely different developmental time periods. Interestingly, in a monocytic cell line, alcohol actually increases GR nuclear translocation (Ng et al., 2017). This emphasizes the fact that alcohol’s effects on GR signaling can be highly cell-type specific.

Finally, it is important to note that the interaction between alcohol exposure and GR signaling is not a one-way street. Studies in human adolescents have demonstrated that polymorphisms in the NR3C1 gene (which encodes GR) are associated with early onset of alcohol con-
sumption and drunkenness in young teens (Desrivières et al., 2011). Likewise, polymorphisms in the FKBP5 gene (which encode FKBP51 that downregulates GR nuclear translocation) have also been associated with increased alcohol consumption in humans (B. Qiu et al., 2016). It is therefore important to better characterize the functional effects of these naturally-occurring polymorphisms on GR intracellular signaling dynamics.

**Novel glucocorticoid receptor interactions**

Finally, because of the dearth of information regarding the molecular mechanisms of GR-mediated CRF repression, chapter VI investigated the neuronal GR interactome in the context of agonist (DEX) or vehicle treatment. We preliminarily identified 59 putative GR-interacting proteins, 16 of which were highly associated with BioID-GR transfection, and the majority of which were novel possible interactions. Of the proteins identified, three (BRG-1, ARID1B, and SMARCC2) had previously been demonstrated to interact directly or indirectly with GR as part of the nBAF complex (Hsiao et al., 2003; Hurlstone et al., 2002; Kadam et al., 2000). However, it is so far unknown if this gene regulatory complex would specifically influence CRF gene expression in the context of HPA axis function. Of the novel putative GR-interacting proteins we identified, a few stand out as logical primary candidates for validation and further investigation as they are localized in the nucleus and are known to influence transcription or splicing, including Sp-100 and CDC5L.

Our study was the first to attempt to characterize the GR interactome in a neuron-like cell line, but a handful of other studies have attempted to characterize the effects of glucocorticoids on the proteome of various other cell types. One study of liganded and unliganded GR isolated from rat liver lysates by immunoprecipitation that was then subject to 2D-DIGE and identification of differentially bound proteins by mass spectrometry identified several proteins known to
interact with GR (i.e., HSP90, FKBP51, etc.); however, our observed GR interactome had very little overlap with what had been reported in that study (Hedman et al., 2006). These discrepancies are likely due to different experimental techniques as well as the completely different cell types used in the two studies. Other studies have simply investigated the proteomic effects of glucocorticoid treatment (not necessarily in conjunction with purification of GR-interacting proteins) in mouse glomerular podocytes or the THP-1 monocytic cell line (Billing et al., 2007; Ransom, Vega-Warner, Smoyer, & Klein, 2005). Again, while there was little overlap between our study and these two, one protein of interest identified in both of the abovementioned studies, as well as our study to a lesser degree of confidence, was the actin binding protein, gelsolin. Gelsolin is of particular interest because it has been shown to interact with other nuclear receptors and act as a transcriptional coregulator; furthermore, our laboratory has demonstrated that the interaction of gelsolin with ERβ changes in response to age and estrogen deprivation (Mott et al., 2014; Nishimura et al., 2003). Should the GR-gelsolin interaction be validated in the neuronal system, the mechanisms by which this interaction is altered might be of interest due to its likely functional importance. Collectively, these studies highlight the cell-specific nature of the effects of glucocorticoids and GR signaling, emphasizing the need to better characterize GR protein:protein interactions and their ultimate effect on function in various tissues.

**Future Directions.**

The work presented here contributes significantly to our collective understanding of GR signaling in neuronal cells, how adolescent alcohol may impact that signaling, and ultimately the behavioral and neuroendocrine consequences of adolescent binge alcohol exposure. Together, these data generally support the hypothesis that adolescent binge alcohol exposure alters GR sig-
naling leading to long term behavioral and neuroendocrine dysfunction. However, the results of these studies open the doors for many new lines of investigation.

The studies described in Chapters III and IV demonstrate that a history of adolescent binge alcohol exposure, combined with adult acute or chronic stress, leads to an anxiety-like behavioral phenotype and HPA axis dysfunction. However, repeated stress is also a well-known factor in the development of depression, and adolescent alcohol consumption is most closely associated with alcohol use disorders (Naughton et al., 2014; Viner & Taylor, 2007). Further studies should investigate the role of adolescent binge alcohol exposure in the development of a depressive-like phenotype, for example using the forced swim or sucrose preference behavioral tests. It would be prudent to do so both in the context of adolescent binge alcohol exposure alone, and in combination with other adulthood stressors, as we did here for anxiety-like behaviors. It is expected that animals with a history of adolescent binge alcohol exposure would similarly exhibit increased depressive-like behaviors, particularly in combination with chronic stress exposure during adulthood. Additionally, the role of adolescent binge alcohol exposure in the development of addictive behaviors should be investigated. While this line of research is active in many labs, each particular animal model has slightly different effects on behavior. Therefore, it is important to determine if this model results in an increased propensity toward spontaneous alcohol or other drug consumption, or if it lowers the threshold for the development of addictive behaviors. Furthermore, it is important to characterize if this is specific to the development of alcoholism, or if adolescent binge alcohol exposure might also increase the risk of developing addiction to other drugs, and if so, which drugs.

The results described in Chapters V and VI, in combination with previous studies from our laboratory, ultimately address the mechanisms underlying the effects of adolescent binge al-
cohol exposure. We have previously demonstrated that the adolescent alcohol exposure results in hypersensitivity of the HPA axis due to decreased glucocorticoid feedback inhibition in the hypothalamus, and that alcohol exposure reduces the ability of GR to bind the CRF promoter and downregulate CRF promoter activity (Przybycien-Szymanska, Mott, & Pak, 2011; Przybycien-Szymanska, Mott, Paul, et al., 2011). Chapter V demonstrated that this GR dysfunction is not due to dysregulated chaperone binding or major changes to its ability to translocate to the nucleus; rather, alcohol seems to specifically alter its regulation of genes relevant to HPA axis sensitivity, at least acutely after alcohol administration. Chapter VI therefore examined the GR interactome using a proximity-dependent biotinylation screen, and ultimately identified known and novel GR-interacting proteins, many of which also reside in the nucleus and may act as transcriptional coregulators. These studies lay the groundwork for future investigation into the mechanisms underlying glucocorticoid feedback inhibition of CRF expression under normal physiological conditions and in response to adolescent alcohol exposure. The immediate next steps involve improving the screening techniques used and validation of the identified, putative GR-interacting proteins. A logical method to validate these interactions is by adapting the GR co-immunoprecipitation techniques used in Chapter V to investigate the interactions of GR with each identified protein “hit,” preferably in combination with knock-down experiments to ensure specificity of the antibodies. Once these interactions are validated, they should be further characterized to determine if the interactions are relevant to GR’s ability to regulate CRF expression and which other genes might be regulated by GR’s interactions with these proteins. A targeted way to address this (in the context of one or a handful of promoters) would be to use reporter gene assays driven by the promoter of interest. A broader way to address this would involve more complicated, high-throughput assays, such as combinatorial ChIP-seq experiments, or other
techniques depending on which regulatory process is hypothesized to be affected by the validated protein:protein interactions.

It is also important to characterize how differential protein:protein interactions among GR and its novel interacting partners come about, and how these interactions might change GR’s ability to interact with other molecules within the cell. For example, GR is known to be post-translationally modified at several residues; primarily, GR phosphorylation has been studied, but other GR modifications (i.e. SUMOylation) have recently been demonstrated to have functional consequences as well. It would be important to address whether any of these modifications alter the ability of GR to interact with its binding partners. For example, in the case of GR phosphorylation sites, phospho-mimetic and phospho-null mutants can be utilized in conjunction with co-immunoprecipitation experiments (or other techniques to assess protein:protein interactions) to address these types of questions. However, other post-translational modifications can be more difficult to measure or modify experimentally. Furthermore, it would be interesting to note if the identified protein:protein interactions influence GR’s ability to bind DNA, or if these interactions change GR’s preferred DNA sequence that it may bind. Additionally, as some of the novel GR interacting proteins identified are known to regulate mRNA splicing, it would be intriguing to investigate if GR indeed has a role in regulating splicing, or even the processing of other, non-coding RNAs.

The possible new lines of investigation brought on by this work in conjunction with recent studies that demonstrate the cell- and context-specific nature of GR signaling is essentially endless. Because of the relative difficulty of studying molecular pathways in the neuronal system, particularly large gaps in our knowledge remain regarding GR’s signaling in PVN neurons, as well as in other brain regions which exert neuronal control over the HPA axis. While this work
has addressed some of the unknowns in this regard, there is still much work to be done in order to fully comprehend the complexities of this system. Better characterization of these pathways will ultimately lead to better treatment options for the effects of adolescent binge alcohol use, and likely for the treatment of mood disorders in general.

**Clinical Implications.**

The investigation of drugs that modulate HPA axis activity for the treatment of mood disorders has fallen out of popularity in recent years; however, the data presented herein suggest that the right therapeutics, delivered in a more specific manner, may have potential clinical impact, particularly in individuals with a history of adolescent binge drinking. At very least, tests that assess HPA axis dysfunction could be a helpful tool in diagnosing mental health disorders and potentially guiding which therapeutic options are best for patients, although these tests would likely need to be used in conjunction with standard diagnostic tools as they have thus far not been specific in their ability to diagnose mood disorders alone. Furthermore, although the research here did not examine the effects of exogenously administered glucocorticoids, the mechanistic investigation into the neuronal GR interactome could shed some light on the side effects associated with glucocorticoid treatment.

Interestingly, antagonism of GR with mifepristone has been demonstrated in animal models of alcoholism and in human alcoholics to decrease alcohol-seeking behaviors (L. F. Vendruscolo et al., 2012; Leandro F. Vendruscolo et al., 2015). This is likely because stress is known to increase alcohol- (or other drug-) seeking behaviors and blocking the stress response may therefore decrease the stress-induced urge to consume alcohol (Tunstall, Carmack, Koob, & Vendruscolo, 2017). However, it would be ideal to formulate a therapy that could prevent the transition from subclinical drinking behaviors to full-blown alcohol dependence, particularly in
individuals with a history of binge drinking during the teen years. Because our laboratory’s data suggest that GR-mediated HPA axis negative feedback is dysfunctional, it is unclear if GR antagonist treatment would make matters better or worse.

In the last 15 years, a push to create GR-directed drugs with fewer side effects has been made with the intention of treating inflammatory conditions like rheumatoid arthritis, preventing the rejections of transplants, and treating certain blood cancers. This ultimately led to the synthesis of SEGRAMs (selective glucocorticoid receptor agonists/modulators), most of which at this point abolish GR-mediated transactivation while retaining transrepression abilities, therefore retaining the immunosuppressive effects of GR signaling without the unwanted risk of hyperglycemia, skin atrophy, and other side effects (Sundahl, Bridelance, Libert, De Bosscher, & Beck, 2015). More recent research is also targeted at creating SEGRAMs that selectively promote monomeric or dimerized GR states to expand the repertoire of drugs that can modulate GR signaling (De Bosscher, Beck, Ratman, Berghe, & Libert, 2016). Thus far, however, there are no clinically available SEGRAMs approved for use in this country. Because many SEGRAMs promote transrepression of gene expression, it is likely that these drugs would be beneficial in downregulating CRF expression, thereby blunting the HPA axis responsiveness in individuals with a hypersensitive response to stress (for example, in individuals with a history of teen binge drinking). Without target drug delivery, however, this might also produce peripheral effects, particularly on immune function, which in this case would be an unwanted side effect. Furthermore, it is possible that alcohol alters GR (or its interacting proteins) in such a way that its dysfunction could not be overcome by these ligands; for example, if GR is aberrantly post-translationally modified, it may be resistant to the effects of SEGRAMs.
Due to its almost ubiquitous expression and pleiotropic effects, one could argue that GR itself is not the most attractive therapeutic target. By identifying novel GR-interacting proteins (as we have done here), it is possible that particular GR signaling pathways could be better modulated by targeting the particular coregulators, posttranslational modifying enzymes, splicing factors, etc., with which GR interacts, or by interfering with the surfaces on either GR or one of its interacting partners which allow for these protein:protein interactions to occur. The development of such drugs is completely dependent on gaining a better understanding of GR protein:protein interactions in a cell-, context-, and promoter- specific manner. Collectively, this highlights the necessity of basic research into the mechanisms involved in GR signaling.

**Take Home Message.**

Teenage binge drinking impacts the structural development of the brain and has functionally been shown to result in decreased learning and memory capabilities, poor decision making, and increased susceptibility to mood disorders. Animal models of adolescent binge alcohol exposure have demonstrated that alcohol plays a causative role in these structural and functional changes, and furthermore allow for the study of the molecular mechanisms behind these changes. Repeated binge alcohol exposure during adolescence results in long term changes in HPA axis function, rendering it hypersensitive to stressors due to deficient glucocorticoid feedback inhibition. The work presented here demonstrates that adolescent binge alcohol exposure can alter the behavioral responses to acute and chronic stressors during adulthood, as well as induce changes to HPA axis function, suggesting HPA axis dysfunction likely plays a role in dysfunctional behavioral responses to stress. The glucocorticoid receptor is widely implicated in mediating the negative feedback of glucocorticoids on the hypothalamus. However, repeated binge alcohol exposure does not seem to alter global glucocorticoid receptor signaling in hypothalamic cells or
tissue samples. Rather, adolescent binge alcohol exposure appears to selectively alter signaling pathways involved in modulating HPA axis activity. Because of the relative lack of information regarding glucocorticoid receptor-mediated feedback inhibition in the hypothalamus, we also sought to characterize the GR interactome in a neuronal-like cell line. We identified 59 BioID-GR associated proteins, 16 of which were high-confidence “hits.” These included members of the nBAF complex, some of which had previously been shown to interact with GR, while the majority of the identified proteins were novel putative GR-interacting partners. Taken together, the work presented here lends support to the hypothesis that adolescent binge alcohol consumption alters GR function, which results in HPA axis hypersensitivity, which contributes to an increased propensity toward the development of mood disorders. Collectively, this provides rationale for targeting the glucocorticoid receptor and its regulation of the HPA axis in the prevention or treatment of mental health disorders, particularly in those with a history of teenage binge drinking.
CHAPTER VIII
GENERAL METHODS

Chapter III.

Ethics Statement

All animal protocols were approved by the Loyola University Medical Center Institutional Animal Care and Use Committee (IACUC) permit #2013034. All measures were taken to minimize animal numbers and suffering.

Animals

Male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (post-natal day [PND] 25) and allowed to acclimate for five days after arrival. Animals were pair-housed on a 12:12 light/dark cycle with lights on at 7:00h. Food and water were available ad libitum.

Experimental Paradigm

Repeated binge alcohol exposure. After acclimation to the housing environment, beginning on PND 30, animals were handled for 5 min. once per day for seven days by the same individual, between 09:30 and 11:00 hrs. Pubertal binge ethanol (EtOH) treatments commenced on PND 37, which is defined as peri-puberty in this species (Ketelslegers et al., 1978; Södersten et al., 1977). Animals were randomly assigned to either 1) binge EtOH treated (n=20), or 2) water treated control (n=20) groups. The binge EtOH treated animals received 3g/kg ethanol (20% v/v in water) intragastrically (i.g.) via oral gavage once per day at 10:00h for three consecutive days,
then an equivalent volume of water i.g. for two days, then an additional three days with EtOH. This once/day (total of eight days) binge paradigm has been used previously to mimic the pattern of binge alcohol consumption in adolescents (Lauing, Himes, Rachwalski, Strotman, & Callaci, 2008; Przybycien-Szymanska et al., 2010). Our previous studies, and others, have demonstrated that this dose and method of EtOH delivery resulted in blood alcohol concentrations (BAC) between 150-180 mg/dL one hour after the last dose, and does not interfere with normal growth rates or feeding behavior, nor does it result in overt alcohol withdrawal symptoms (i.e. seizure activity) (Callaci et al., 2004; Prins, Przybycien-Szymanska, Rao, & Pak, 2014; Przybycien-Szymanska et al., 2010; Walker & Ehlers, 2009). Furthermore, the calories from alcohol administered are >2% of the rats’ daily caloric intake (average lab rat daily energy consumption is ~100 kcal/day per 100g body weight (Abdoulaye et al., 2006), and a 263g rat receives only 5 kcal from the 3g/kg dosage), therefore it was unnecessary in this model to control for the calorie content of alcohol administered. The water treated control group received eight days of an equivalent volume of water i.g via oral gavage.

**Acute stress paradigm.** After pubertal binge alcohol treatments, both groups of animals were left undisturbed for three weeks (see Fig. 3). At the end of this three week period, the animals were again handled 5 min. once per day for seven days, as described above. At 10:00h on PND 73, prior to further manipulation, animals were given a trial test in the elevated plus maze (EPM) to establish a baseline level of anxiety-like behavior. Then, animals within each group were randomly assigned to either a) a restraint stress group (n=10 per group), or b) an unstressed control group (n=10 per group). Next, on PND 74 at 09:30h, animals in the restraint stress group were placed in a plastic rodent restraint tube (Stoelting Co. #51335) inside a fresh cage for 30 min., then 5 min. after being removed from the restraint tube, the rats were tested in the EPM again,
and 5 min. after ending the EPM test, the animals were euthanized. It has been previously demonstrated that plasma corticosterone (CORT) levels reach a peak after 30 min. inside a plastic restraint tube (Cole et al., 2000). Animals in the unstressed control group were placed singly in a fresh cage for 35 min, then tested in the EPM again, and 5 min after ending the EPM test, the animals were euthanized. Refer to Figure 3 for schematic of experimental paradigm.

**Elevated Plus Maze Testing**

Elevated Plus Maze testing was conducted with the Rat Elevated Plus Maze apparatus (Stoelting Co. #60240) and recorded using a video camera and ANY-maze software (Stoelting Co.). Testing was conducted in a dimly-lit room (~5 lux) with white noise generated by a HoMedics Sound Spa Relaxation machine (~70dB, equivalent to the white noise generated by the HVAC system in the animal housing room). Rats were placed singly in the center of the maze facing an open arm by a female experimenter, marking the beginning of the test period. The rat was then allowed 5 min. to explore the maze freely, after which the recording stopped automatically, and then the rat was returned to its cage. Later, both spatiotemporal and ethological analyses were conducted using the recorded videos and ANY-maze.

**Elevated plus maze analysis.** All parameters were analyzed from the video recordings by an investigator blinded to the animal treatment paradigms. For the spatiotemporal analysis, the maze was divided into three zones, specified using the ANY-maze software: the open arms, the closed arms, and the intersection. In order to be considered in the open or closed arms, at least 80% of the rat’s body surface area had to be inside that zone (consistent with the “four-paw rule”). The rat was considered to be in the intersection if it was not considered to be in either the open or closed arms. The amount of time spent in any given zone was divided by the total test duration to calculate the percentage of time spent in the zone. The total distance travelled and average speed
were also measured to consider differences in overall locomotor activity. For the ethological behavioral analyses, scoring of head dips, stretched attend postures, and rearing behaviors were manually recorded by a blinded trained observer. A head dip was defined as the rat extending its head over the edge of the maze and down toward the floor. A stretched attend posture was defined as the rat extending forward with its front paws, then retracting back to its original position. A rearing was defined as the rat sitting back on its hind paws and elevating its front paws, moving vertically.

_Tissue Processing_

Animals were euthanized humanely by rapid decapitation after anesthesia with inhaled isoflurane. Trunk blood was immediately collected in heparinized tubes on ice, centrifuged at 3000 rpm at 4°C for 10 min., then the plasma was stored at -20°C. Brains were rapidly dissected and flash frozen in isopentane chilled with dry ice, then stored at -80°C. Plasma CORT levels were measured using a Corticosterone ELISA Kit (Enzo Life Sciences #ADI-900-097), both according to manufacturer instructions. Microdissection of the paraventricular nucleus of the hypothalamus (PVN) and ventral hippocampus (V.Hipp) was performed as previously described (Prins et al., 2014; Przybycien-Szymanska, Mott, Paul, et al., 2011). Briefly, brains were sectioned at 200µm using a Leica CM3050 S cryostat, then the specified brain regions were microdissected using a Palkovit’s brain punch tool (Stoelting Co.) and confirmed using The Rat Brain in Stereotaxic Coordinates, Fourth Edition Atlas (G. Paxinos and C. Watson). For the PVN, we microdissected 0.75 mm area on each side of the third ventricle between 0.8 mm and 2.12 mm posterior to Bregma, 8 mm below the top of the brain. For the V.Hipp, we microdissected between 3 mm and 6 mm lateral to the midline, between 4.16 mm and 6.05 mm posterior to Bregma, 3 mm below the top of the brain and 2 mm above the bottom of the brain. Brain tissue punches were stored at
-80°C; later, genomic DNA, RNA, and protein were isolated using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen #80004), according to manufacturer instructions. This kit first removes genomic DNA by column centrifugation before the separation of RNA and protein.

**RT-qPCR**

Total RNA (250-350 ng) was reverse transcribed using the First-Strand Synthesis SuperMix for RT-qPCR (Invitrogen). cDNA products were treated with RNase H (Promega). PCR was performed in triplicate using iTaq™ Universal SYBR® Green Supermix and the following primers:

- *rat AVP forward*: 5’-GGGCAGGTTCTTCCCTGC-3’, *rat AVP reverse*: 5’-CACCTCTGCTGCTACT-3’;
- *rat cFos forward*: 5’-AGCATGGGCTCCCCTGTCA-3’, *rat cFos reverse*: 5’-GAGACCAGAGTGGGCTGCA-3’;
- *rat CRF forward*: 5’-GAGAAAGGGAAAGGCAAAG-3’, *rat CRF reverse*: 5’-ATCAGAATCGGCTGAGGGT-3’;
- *rat GR forward*: 5’-CACCCATGATCCTGTCAGTG-3’, *rat GR reverse*: 5’-AAAGCCTCCCTGCTAACC-3’;
- *rat HPRT forward*: 5’-GTTCTTTTGTGACCTGCTGGAT-3’, *rat HPRT reverse*: 5’-CCAACACTTCCAGAGGTCTCCTTT-3’. All primer sets were intron-spanning, with the exception of the GR primer set. RT-negative control reactions were performed to ensure that there was no interfering genomic DNA contamination. All samples were normalized to the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) housekeeping gene, as it is not altered by EtOH treatment (Przybycien-Szymanska et al., 2010), and transcript fold changes were calculated using the ΔΔCt method (Livak & Schmittgen, 2001).

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism 7. The EPM pre-test data (baseline, trial one) were analyzed using unpaired t-tests (n=20 per group). The biochemical (ELISA, qRT-PCR) and EPM post-test (after acute stressor, trial 2) data were analyzed by two-way ANOVA,
with Sidak’s multiple comparisons post-hoc tests (n=10 per group, except PVN cFos mRNA, where n=7-9 per group due to sample exhaustion). In all cases, p<0.05 was considered significant.

Chapter IV.

Animals

As in Chapter III, male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (post-natal day [PND] 25) and allowed to acclimate for five days after arrival. Animals were pair-housed on a 12:12 light/dark cycle with lights on at 7:00h. Food and water were available ad libitum.

Experimental Paradigm

Repeated binge alcohol exposure. The repeated binge alcohol exposure paradigm was conducted on PND37-44 as described in the methods for Chapter III, with 18 animals per group (36 total).

Chronic stress paradigms. After pubertal binge alcohol treatments, both groups of animals were left undisturbed for two weeks (see Fig. 8). At the end of this two week period, the animals were again handled 5 min. once per day for seven days, as described above. Then, animals within each group were randomly assigned to either a) a repeated restraint stress group (n=6 per group), b) a repeated binge EtOH group (n=6 per group), or c) an unstressed control group (n=6 per group).

Next, on PND 67 at 09:30h, animals in the repeated restraint stress group were placed in a plastic rodent restraint tube (Stoelting Co. #51335) inside a fresh cage for 30 min., then returned to their home cage; this was repeated daily through PND 74, for a total of eight days. For the animals in the repeated binge EtOH group, these animals underwent another three-day EtOH, two-day water, three-day EtOH repeated binge alcohol exposure (as described above) from PND 67-74. For the animals in the unstressed control group, animals were simply handled for 5 min. per day from
PND 67-74. On PND 75 at ~10:00h (24 hours after the last stressor), all of the rats were tested in the EPM, and 5 min. after ending the EPM test, the animals were euthanized. Refer to Figure 8 for schematic of experimental paradigm.

*Elevated Plus Maze Testing*

Elevated Plus Maze testing was conducted as described in the methods for Chapter III.

*Tissue Processing*

Euthanasia, plasma CORT measurements, and brain microdissection and DNA/RNA/protein isolation were conducted as described in the methods for Chapter III.

*RT-qPCR*

RT-qPCR for HPRT, CRF, AVP, and GR were conducted as described in the methods for Chapter III.

*Statistical Analysis*

Statistical analyses were performed using GraphPad Prism 7. The biochemical (ELISA, qRT-PCR) and EPM data were analyzed by two-way ANOVA, with Sidak’s multiple comparisons post-hoc tests (n=6 per group). In all cases, p<0.05 was considered significant.

**Chapter V.**

*In vitro repeated EtOH and DEX treatment*

Rat PVN-derived IVB cells were cultured in DMEM supplemented with 10% FBS (growth media). Cells were routinely tested for the presence of mycoplasma contamination using the Mycosensor PCR Assay Kit (Agilent), and discarded if tested positive. 24 hours after plating 10^5 cells per 10-cm culture dish, the culture media was changed to DMEM+10% charcoal-stripped FBS (stripped media). The next day, cells were treated with 100nM dexamethasone, 50mM ethanol, both, or vehicle, diluted in stripped media for two hours inside air-tight chambers. On the
first two treatment days, the media was then replaced with fresh stripped media. On the third day, the cells were immediately lysed after treatment.

Cytoplasmic and nuclear protein fractionation

Cytoplasmic and nuclear protein fractionation was conducted using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (catalog #78835) according to manufacturer protocol. Briefly, after the third day of cell treatment, the culture dishes were washed 3x with cold PBS, then cells were scraped in PBS into a 15mL conical tube and centrifuged at 1000g for 3 min. to pellet the cells. Then, the PBS supernatant was removed, and the pellet was resuspended in CER-I buffer with protease and phosphatase inhibitor cocktail (Pierce catalog #88668). After incubating 10 min. on ice, CER-II buffer was added, then the samples were vortexed and centrifuged to pellet the nuclei (the supernatant was saved as the cytoplasmic protein fraction). Nuclei were resuspended in NER buffer with protease and phosphatase inhibitors, vortexed 15 sec. every 10 min. four times (40 min. total), then centrifuged (supernatant was saved as nuclear protein fraction). Protein concentration was measured by BCA assay (Pierce catalog #23225).

Western blotting

25µg total protein from each sample was boiled at 95°C for 5 min. with 4x Laemmlı sample buffer, resolved on an 8% SDS-PAGE gel for ~one hour at 120V, then transferred to a 0.045µm PVDF membrane for one hour at 100V. Total protein staining was conducted using LI-COR REVERT Total Protein Stain (catalog #926-11011) according to manufacturer instructions, then imaged on a LI-COR Odyssey. After destaining, membranes were blocked for one hour at room temperature with Odyssey blocking buffer (LI-COR catalog #927-50000) diluted 1:1 with 1X TBS, then incubated in primary antibody solution diluted in Odyssey blocking buffer 1:1 with 1X TBST (0.1% Tween) overnight at 4°C. The GR primary antibody used was IA-1 (a generous
gift from Miles Pufall), diluted 1:2500. The next day, membranes were washed 2x10 min., then incubated with the appropriate secondary antibody diluted in Odyssey blocking buffer 1:1 with 1X TBST (0.1% Tween) for one hour at room temperature. The 680RD-conjugated anti-rabbit secondary antibody was diluted 1:10,000. Membranes were washed 2x10 min. before imaging on a LI-COR Odyssey (laser intensity for both 700 and 800 channels was 5.0). Densitometry was performed using LI-COR Image Studio Lite version 5.2, and protein bands of interest were normalized to total protein staining. To calculate the proportion of nuclear GR, the normalized GR intensity in the nuclear fraction was divided by the sum of the normalized GR intensity in the cytoplasmic and nuclear fractions, using the formula:

\[
Proportion \, nuclear \, GR = \frac{(\text{nuclear GR} + \text{nuclear total protein})}{((\text{nuclear GR} + \text{nuclear total protein}) + (\text{cytoplasmic GR} + \text{cytoplasmic total protein}))}
\]

Animals

As in Chapters III and IV, male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (post-natal day [PND] 25) and allowed to acclimate for five days after arrival. Animals were pair-housed on a 12:12 light/dark cycle with lights on at 7:00h. Food and water were available ad libitum.

Experimental Paradigm

Repeated binge alcohol exposure. The repeated binge alcohol exposure paradigm was conducted on PND37-44 as described in the methods for Chapter III, with 16 animals per group (32 total). Animals were euthanized one hour after the last dose of EtOH or water on PND 44.

Tissue processing

One hour after the last dose of alcohol or water, animals were euthanized and tissue was collected and microdissected as described in the methods for Chapter III. Tissue punches were sus-
pended in 400µL cold co-IP buffer (0.1% CHAPS, 40 mM HEPES, pH 7.5, 120 mM NaCl, 1mM EDTA, 10mM Na Pyrophosphate and 10mM β-glycerophosphate; supplemented with protease and phosphatase inhibitor cocktail), homogenized using a hand-held homogenizer, vortexed briefly, frozen and thawed three times, then centrifuged at 12,000 rpm for 20 min. at 4°C (supernatant was collected). Protein concentration was measured by BCA assay (Pierce catalog #23225).

**Co-immunoprecipitation**

600µg of total protein per sample was incubated with 2µg of antibody overnight at 4°C with end-over-end mixing. The GR antibody used was IA-1 (from Miles Pufall), or the control antibody was normal rabbit IgG (Millipore). The next day, 50ul of washed bead slurry (Millipore PureProteome Protein A/G Mix Magnetic Beads, catalog #LSKMAGAG10) was added to each antibody:antigen complex and incubated 1 hour at room temperature with end-over-end mixing. The beads were washed three times with co-IP buffer, then the proteins were eluted with 2X Laemmli sample buffer by heating at 70°C for 10 min. After transferring samples to a new tube, samples were boiled at 95°C for 5 min., then run alongside 10% input samples on an 8% SDS-PAGE gel. Proteins were then transferred to a 0.045µm PVDF membrane for 1 hour at 100V. Total protein staining was conducted using LI-COR REVERT Total Protein Stain (catalog #926-11011) according to manufacturer instructions, then imaged on a LI-COR Odyssey. After destaining, membranes were blocked for 1 hour at room temperature with Odyssey blocking buffer (LI-COR catalog #927-50000) diluted 1:1 with 1X TBS, then incubated in primary antibody solution diluted in Odyssey blocking buffer 1:1 with 1X TBST (0.1% Tween) overnight at 4°C. The GR primary antibody used was IA-1 (a generous gift from Miles Pufall), diluted 1:2500, and the HSP90 antibody was from Abcam (catalog #ab1429), diluted 1:400. The next day, membranes were
washed 2x10 min. with TBST, then incubated with the appropriate secondary antibodies diluted in Odyssey blocking buffer 1:1 with 1X TBST (0.1% Tween) for 1 hour at room temperature. The 680RD-conjugated anti-rabbit secondary antibody and 800CW-conjugated anti-mouse secondary antibody were both diluted 1:10,000. Membranes were washed 2x10 min. before imaging on a LI-COR Odyssey (laser intensity for both 700 and 800 channels was 5.0). Densitometry was performed using LI-COR Image Studio Lite version 5.2, and protein bands of interest were normalized to total protein staining.

**RT-qPCR**

Total RNA isolation was performed on sonicated tissue samples using Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacturer’s directions. Reverse transcription was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen catalog #18080-51). PCR was performed in triplicate using FastStart Universal SYBR Green Mastermix (Roche catalog #04913914001) and the following primers: rat FKBP5 forward 5’-CGG-GATGTGGTGTTCGTACAT-3’, rat FKBP5 reverse 5’-TTCTTTGGCTTCTTTTCGAAGCTC-3’, rat HPRT forward 5’-GTTCTTTGCTGACCTGCTGCTTGAT-3’, rat HPRT reverse 5’-CCAACACCTCAGAGGTCTCCTTT-3’, rat PER1 forward 5’-GTGCATCTCAGCTCAAGAT-3’, rat PER1 reverse 5’-CACTGGTAGACGGGGTTGCC-3’. All primer sets were intron-spanning, with the exception of the GR primer set. RT-negative control reactions were performed to ensure that there was no interfering genomic DNA contamination. All samples were normalized to the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) housekeeping gene, as it is not altered by EtOH treatment (Przybycien-Szymanska et al., 2010), and transcript fold changes were calculated using the ΔΔCt method (Livak & Schmittgen, 2001).
Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7. The co-immunoprecipitation data were analyzed using unpaired t-tests (n=7 per group for hypothalamus, n=12 and 16 for dorsal hippocampus control and EtOH, respectively). The in vitro GR nuclear localization data were analyzed by two-way ANOVA, with Sidak’s multiple comparisons post-hoc tests (n=6 per group). In all cases, p<0.05 was considered significant.

Chapter VI.

Constructs and cloning

The pEGFP-hGR (#47504) and pcDNA3.1-mycBioID (#35700) constructs were purchased from Addgene (Cambridge, MA). The pcDNA3.1-mycBioID-hGR construct was created using Gibson Assembly. Briefly, the NR3C1 gene (hGR) was amplified by PCR with Q5 High-Fidelity DNA polymerase (NEB) from the pEGFP-hGR construct with the primers 5'ggagaaatctecctgagaagcgcagagaagatggactccaaagaatcattaactcct-3' and 5'caacagatggtgcaactacaagcagctacttttgatgaaacgaagttttattatattcc-3'. The pcDNA3.1-mycBioID construct was linearized and amplified by PCR with Q5 DNA polymerase and the primers 5'-accaggagttatgtctttggagctcatctttcttgcttcggcttcaggg-3' and 5'-atcaaaaaactctttctcatcaaatgctgtgctcttagtggtgcgag-3'. After ensuring that the PCR products were a single band (by running a sample on a 1% agarose gel), the PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega), then combined in a 7:1 insert : vector molar ratio (in 10µL ddH2O) mixed with 10µL 2X Gibson Assembly Master Mix (NEB), and incubated at 50°C for 1 hour. 2µL of the Gibson Assembly product was directly used for transformation into competent E. coli (NEB 5α). Colonies were then subject to DNA mini-prep and
the resulting construct DNA was sequenced with the primer 5’-ggctcctacctgagcagat-3’ to ensure correct product sequence.

**Cell culture**

HEK293T cells were cultured in DMEM supplemented with 10% FBS (HEK growth media). SK-N-SH cells were cultured in MEM+Earles salts supplemented with 1% L-glutamine, 1% non-essential amino acids, and 10% FBS (SK-N-SH growth media). Both cell lines are commercially available and have been widely characterized. Cells were routinely tested for the presence of mycoplasma contamination using the Mycosensor PCR Assay Kit (Agilent), and discarded if tested positive. Cells were plated at 10^5 cells per 10-cm culture dish for small scale experiments (Western blotting) or 2x10^5 cells per 150-mm culture dish for large scale experiments (LC-MS/MS). When the cells reached ~50% confluence, the culture media was changed to “stripped media,” containing all the components of the appropriate growth media, with the replacement of FBS with charcoal-stripped FBS. The next day, cells were treated with 100nM dexamethasone or vehicle (0.001% EtOH) in stripped media supplemented with 50µM biotin. After 24 hours of treatment, cells were lysed with RIPA buffer (for pull-down, Western blotting, and/or LC-MS/MS), or subject to cytoplasmic and nuclear protein fractionation as described in the methods for Chapter V (only for Western blotting).

**Streptavidin bead pull-down**

Pull-downs were performed generally as described in Le Sage, et al. (2016). RIPA lysates were incubated with streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen) at a ratio of 500µg total protein : 100 ul bead slurry overnight at 4°C with end-over-end mixing. The following day, the supernatants were removed, then the beads were washed for 8 min. with buffer 1 (2% SDS in ddH2O), then 8 min. with buffer 2 (50mM HEPES pH 7.5,
500mM NaCl, 1mM EDTA, 0.1% deoxycholic acid, 1% Triton X-100), then 8 min. with buffer 3 (10mM Tris HCl pH 7.4, 250mM LiCl, 1mM EDTA, 0.1% deoxycholic acid, 1% NP-40). Beads were briefly washed with 50mM Tris pH 7.4 to remove detergents, before elution of biotinylated proteins with 1X Laemmli sample buffer with boiling at 95°C for 5 min. Supernatants were then resolved by SDS-PAGE, then either used for further Western blotting (as described in Chapter V), or SDS-PAGE gels were cut for further LC-MS/MS sample prep. For LC-MS/MS, technical duplicates of each sample were run side-by-side, then pooled prior to SDS-PAGE. For Western blotting, primary antibodies used were mouse anti-myc (Proteintech catalog #66004-1-Ig, diluted 1:10,000), mouse anti-actin (Proteintech catalog #66009-1-Ig, diluted 1:5000), IA-1 rabbit anti-GR (generous gift from Miles Pufall, diluted 1:2500), or rabbit anti-HDAC-1 (Abcam catalog #ab19845, diluted 1:5000).

Preparation of samples for mass spectrometry-based proteomics analysis

The gel bands of interest were excised by new razor blades with clean surfaces and further reduced by cutting the size of gel pieces to 1 – 2mm in each dimension to optimize peptide recovery and minimize peptide loss. After excision, the proteins present in gel pieces were washed twice with HPLC-grade water (Optima LCMS, Fisher Chemical, USA) and 1:1 v/v of 0.1M NH₄HCO₃/H₂O for 15 min. each with agitation. The washing solution was then removed completely and enough HPLC-grade acetonitrile (ACN, Optima LCMS, Fisher Scientific, USA) was added to cover the gel pieces. All the solvent volumes used in the washing steps were roughly equal to twice the gel volume. After the gel pieces shrunk and stuck together, the ACN was removed and the gel pieces were rehydrated in 0.1M NH₄HCO₃ for 10 min. An equal volume of ACN was then added to finally get 1:1 v/v of 0.1M NH₄HCO₃/ACN.
After incubation, removing all liquid, and drying down the gel pieces in a vacuum centrifuge, proteins were reduced with 10mM dithiothreitol and alkylated with 55mM iodoacetamide in 0.1M NH₄HCO₃. After reduction and alkylation, gel pieces were washed as described above. Following tryptic digestion (Pierce Trypsin Protease, MS grade), with a ratio of 1:20 trypsin : analytes, for 24 hours at 37°C, the peptides were recovered and extracted from the gel pieces by addition of a 10µL of 25mM 0.1 M NH₄HCO₃ and 5% formic acid and ACN (5µL of each). Pooling and drying down all the extracts, the tryptic peptides were dried and re-dissolved in 10µL of 95% ACN with 5% formic acid and let it sit for 10 min. in preparation for the LC-MS/MS analysis.

Identification of proteins with LC-MS/MS

Tryptic digests of proteins from the gel bands were first purified and enriched by a pre-column (C18 PepMap 100, 5µm, 100 Å, 300µm i.d. x 5mm, Thermo Scientific, USA) at the flow rate 30µL/min. for 5 min. and then separated by a analytical reversed-phase column (C18 Acclaim PepMap 100, 75µm x 15cm, nanoViper, Thermo Scientific, USA) at the flow rate 200nL/min. for 66 min. using a linear gradient from 98% solvent A (98% water, 2% ACN, 1% formic acid) and 2% solvent B (2% water, 98% ACN, 1% formic acid) to 35% solvent B over 50 min. The eluted peptides were introduced directly by electrospray into the LTQ Orbitrap mass spectrometer (Thermo Orbitrap Elite, Thermo Scientific, USA).

Data dependent acquisition (DDA) was carried out for LC-MS/MS of digested samples. Each survey scan acquired in the Orbitrap at the mass range 200 – 1600 Da and FT resolution 120000 fwhm was followed by 10 MS/MS scans of the most intense precursor ions in the linear ion trap with enabled dynamic exclusion for 20 sec. The normalized collision energy by collision induced dissociation (CID) was set to 35% and one microscan was acquired for each spec-
The charge state screening was employed to select for ions with at least two charges and rejecting ions with undetermined charge state. CID was triggered when the precursor exceeded 100 ion counts. The ion accumulation time was set to 300 ms (MS) for precursor scan and 50 ms (MS/MS) for product scan.

Data processing

The resulting LC-MS/MS data was analyzed by PEAKS, version 8.0 (Bioinformatics Solutions Inc.) with the *homo sapiens* database downloaded from the Uniprot website (http://www.uniprot.org/). In data refinement, charge status was set as 1 – 4 and filter quality was set as more than 65%. For *de novo* section, error tolerances, 15 ppm for parent mass and 0.5 Da for fragment mass were set to limit false discovery rate (FDR). With chemical modifications, carbamidomethyl cysteine residues were selected as a fixed modification and oxidization of methionine was set as a variable modification. Three max missed cleavages were allowed.

The statistical and probabilistic models using generated ion probabilities of peptide sequences sometimes increase the error of the match number of spectra that are present in the spectral library, so in order to enhance the accuracy of peptide identification, 1% was set as FDR value for peptide match and at least two unique peptides were identified for a protein.

Search results were further refined by excluding proteins which were inappropriately sized for the fraction in which they were identified. For a hit to be considered associated with either or both of the BioID-GR samples, it had to be unique to at least one of the replicates of that condition, or if it was identified in all three biological replicates of a given condition, it was permitted to have been identified in one of the control samples.
APPENDIX A

SUPPLEMENTARY FIGURES
Figure 19: Dot plots depicting anxiety-like behavior under resting conditions. The percent of time in the closed arms (A), intersection (B), and open arms (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=20 per group).
Figure 20: Dot plots depicting anxiety-like behavior following acute restraint stress. The percent of time in the closed arms (A), open arms (B), and intersection (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There was a significant main effect of gavage on time spent in the intersection of the arms (C), and significant main effects of gavage and restraint on stretched attend postures (D). Asterisk (*) denotes significant pairwise comparisons between indicated groups.
Figure 21: Dot plots depicting regional levels of CRF, AVP, and cFos transcripts. mRNA levels of corticotropin releasing factor (A and C), cFos (B and D), and arginine vasopressin (E), in the ventral hippocampus (A and B) and paraventricular nucleus of the hypothalamus (C-E), measured by RT-qPCR relative to the unrestrained H2O group. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There were significant main effects of restraint on V.hipp cFos (B), PVN CRF (C), and PVN cFos (D) levels. Asterisk (*) denotes significant main effect of restraint in panel B and significant pairwise comparisons between indicated groups in panels C and D.
Figure 22: Dot plots depicting plasma CORT and brain region expression of GR. Circulating plasma levels of corticosterone (A), measured by ELISA. mRNA levels of glucocorticoid receptor in the ventral hippocampus (B), and paraventricular nucleus of the hypothalamus (C), measured by RT-qPCR relative to the unrestrained H2O group. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=10 per group). There were significant main effects of gavage and restraint on plasma CORT (A), and a significant main effect of restraint on V. hipp GR mRNA (B). Asterisk (*) denotes significant pairwise comparisons between indicated groups in panel A and significant main effect of restraint in panel B.
Figure 23: Dot plots depicting anxiety-like behavior following adolescent binge alcohol and either adult binge alcohol or adult repeated restraint stress. The percent of time in the closed arms (A), intersection (B), and open arms (C) of the elevated plus maze was calculated by dividing the amount of time spent in the given zone by the total duration of the test (300s). The number of stretched attend postures (D), head dips (E), and rearing behaviors (F), were scored by a blinded observer. The total distance travelled (G), and the average speed (H) were used as indicators of overall motor activity. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA in which p<0.05 was considered significant (n=6 per group).
Figure 24: Dot plots depicting regional levels of CRF, AVP, and GR transcripts. mRNA levels of corticotropin releasing factor (A and B), arginine vasopressin (C), and glucocorticoid receptor (D and E), in the paraventricular nucleus of the hypothalamus (A,C,E) and ventral hippocampus (B and D), measured by RT-qPCR relative to the unrestrained H2O group. Circulating plasma levels of corticosterone (F) were measured by ELISA. Data are expressed as mean ± SEM, and were analyzed by two-way ANOVA with Sidak’s multiple comparisons post-hoc tests, in which p<0.05 was considered significant (n=6 per group). There was a significant main effect of adolescent alcohol on PVN CRF (A).
Figure 25: Dot plot depicting effects of repeated ETOH or DEX on GR nuclear localization. After three days of denoted treatment, IVB cells were lysed and nuclear and cytoplasmic protein fractions were immunoblotted for GR (representative blot shown in (A)). The proportion of nuclear GR was calculated by dividing the relative (to total protein) GR signal protein in the nuclear fraction by the relative GR signal in both cytoplasmic and nuclear fractions for each sample. The summary data are quantified in (B). Data are expressed as mean ± SEM (n=6), and were analyzed by two-way ANOVA, in which p<0.05 was considered significant. There was a significant main effect of DEX treatment on the fraction of nuclear GR.
Figure 26: Dot plots depicting GR-HSP90 interaction in the dorsal hippocampus after adolescent binge alcohol exposure. D.hipp lysates were subject to immunoprecipitation with a GR-specific antibody, then immunoblotted for HSP90 and GR (representative blot shown in (A)). The relative HSP90 signal in the i.p. lanes was calculated and quantified in (B). The relative GR signal in the input lanes was calculated and quantified in (C). The relative HSP90 signal was calculated and quantified in (D). Data are expressed as mean ± SEM (n=12-16), and were analyzed by t-test.
Figure 27: Dot plots depicting GR-HSP90 interaction in the hypothalamus after adolescent binge alcohol exposure. Hypo lysates were subject to immunoprecipitation with a GR-specific antibody, then immunoblotted for HSP90 and GR (representative blot shown in (A)). The relative HSP90 signal in the i.p. lanes was calculated and quantified in (B). The relative GR signal in the input lanes was calculated and quantified in (C). The relative HSP90 signal was calculated and quantified in (D). Data are expressed as mean ± SEM (n=7), and were analyzed by t-test.
Figure 28: Dot plots depicting hypothalamic levels of PER1 and FKBP5. mRNA levels of PER1 (A), and FKBP5 (B), in the hypothalamus was measured by RT-qPCR relative to the control group. Data are expressed as mean ± SEM (n=7), and were analyzed by t-test where p<0.05 was considered significant (*).


Caldwell, K. K., Goggin, S. L., Tyler, C. R., & Allan, A. M. (2014). Prenatal alcohol exposure is associated with altered subcellular distribution of glucocorticoid and mineralocorticoid receptors in the adolescent mouse hippocampal formation. *Alcoholism, Clinical and


Echeverria, P. C., Mazaira, G., Erlejman, A., Gomez-Sanchez, C., Pilipuk, G. P., & Galigniana,


VITA

The author, Audrey Rose (Henning) Torcaso, was born on October 29th, 1990 to Kent and Carole Henning. She attended Carthage College in Kenosha, WI where she earned a Bachelor’s of Arts, magna cum laude, in Biology and Neuroscience in May 2012. After graduation, Audrey matriculated into the Loyola University Chicago Stritch School of Medicine Integrated Program in Biomedical Science, and began her graduate education in the Neuroscience Graduate Program in the laboratory of Dr. Renzhi Han. After passing her qualifying exam in July 2014, Audrey switched laboratories and began her dissertation research under the mentorship of Dr. Toni Pak.

Audrey served as the Graduate Student Council co-president for the 2014-2015 school year. She also participated in Loyola’s Research Mentoring Program in 2015. In April, Audrey won first place in the Midwest Regional Three Minute Thesis competition.

Audrey’s dissertation work on the effects of teenage binge drinking on behavior and HPA axis function was supported by a T32 Institutional Training Grant from the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health. After completion of her graduate studies, Audrey will pursue a Postdoctoral Fellowship in the lab of Dr. Cecilia Hillard at the Medical College of Wisconsin in Milwaukee, Wisconsin.

Audrey currently resides in Kenosha, Wisconsin with her husband, Joe, and their two dogs, Mario and Luigi.