The Dissertation Committee for Elizabeth Joy Crofton Certifies that this is the approved version of the following dissertation:

CELLULAR MECHANISMS OF ENVIRONMENTAL ENRICHMENT: NOVEL DISCOVERY-BASED STRATEGIES FOR TARGET IDENTIFICATION FOR NEUROPSYCHIATRIC DISORDERS

Committee:

Thomas A. Green, Ph.D., Mentor

Giulio Taglialatela, Ph.D.

Jonathan Hommel, Ph.D.

Fernanda Laezza, M.D., Ph.D.

Laura O'Dell, Ph.D.

_____

Dean, Graduate School

CELLULAR MECHANISMS OF ENVIRONMENTAL ENRICHMENT: NOVEL DISCOVERY-BASED STRATEGIES FOR TARGET IDENTIFICATION FOR NEUROPSYCHIATRIC DISORDERS

by

Elizabeth Joy Crofton, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

July, 2017

Dedication

To Mom and Dad,

to MWY,

to GTB,

and to CCC.

Acknowledgements

I would first like to thank Dr. Green for his incredible mentorship, guidance, and scientific expertise. The many awards and publications I have gained throughout graduate school are due to Dr. Green's support, encouragement, and commitment to my success. In one of his earliest support letters, Dr. Green wrote that he learned from his graduate mentor, Dr. Michael Bardo, that regardless of how groundbreaking a scientist's research is today, that research will be outdated in 20 years and completely forgotten in 40. As a result, a scientist's true legacy is not the number of papers published or individual accolades, but rather the people they train. Dr. Green certainly takes this very seriously and is very generous with his time, funds, and advice. He inspires me to be a dedicated mentor as well. I would not be the scientist I am today without Dr. Green and I could not have done this without him. My hope is that Dr. Green continues to use the oxford comma in memory of me.

Thank you to my fantastic committee members, Dr. Giulio Taglialatela, Dr. Fernanda Laezza, Dr. Jonathan Hommel, and my external committee member, Dr. Laura O'Dell, from the University of Texas at El Paso. I very much appreciate their guidance and support.

Thank you to all of the past and present Green Laboratory members, Dingge Li, Xiuzhen Fan, Shyny Koshy, Steve Dragosljvich, but most of all, Dr. Yafang Zhang. Thank you Yafang for your unwavering enthusiasm and encouragement. Thank you for your friendship, your help with experiments, and all of our discussions on our various projects. It would not have been the same without you, nor without your help.

Thank you to all of my wonderful co-authors and collaborators. A special thank you to past and present Laezza Laboratory members. Thank you Dr. Laezza, Dr. Miroslav Nenov, and Dr. Talia James. Thank you to the Molecular Genomics Core at UTMB for the next generation RNA sequencing and the Mass Spectrometry Core of the Biomolecular Resource Facility for the proteomic data, especially Dr. Cheryl Lichti, Dr. Bruce Luxon, Dr. Heidi Spratt, Dr. Fanping Kong, and Mala Sinha.

Thank you to everyone in the UTMB Mental Health Research Group and the UTMB Center for Addiction Research for your support and scientific advice.

Thank you to my amazing cohort of friends who started this journey with me and supported me throughout, especially Dr. Shearer and Dr. Umbaugh.

Thank you to the amazing professors at St. Olaf who sparked my interest not only in neuroscience but also addiction research and supported my decision to attend graduate school, especially Drs. Dickinson, Muir, and McMillan. Um Ya Ya!

Thank you to Dr. Kirsch, who gave me my first experience in a research laboratory, was incredibly supportive, and suggested I attend UTMB.

A special thank you to Dr. Andrew Crofton for all you do for me.

Most of all, thank you to Matt, I could not have done it without you.

CELLULAR MECHANISMS OF ENVIRONMENTAL ENRICHMENT: NOVEL DISCOVERY-BASED STRATEGIES FOR TARGET IDENTIFICATION FOR NEUROPSYCHIATRIC DISORDERS

Publication No._____

Elizabeth Joy Crofton, Ph.D.

The University of Texas Medical Branch, 2017

Supervisor: Thomas A. Green

Neuropsychiatric disorders such as anxiety, depression, and substance use disorders are highly prevalent disorders and immensely costly to society. Treatment options are limited, and cocaine use disorder in particular, has no FDA approved pharmacotherapeutic. These disorders are highly comorbid and are often more severe when they co-occur. Thus, it is critical to determine novel targets for substance use disorders with comorbid mood disorders. In order to identify novel targets, we examine an animal model of the resilience to depression and addiction, environmental enrichment. Environmental enrichment is a non-drug, non-surgical, non-genetic manipulation that produces protective depression and addiction phenotypes. Enriched rats are reared in a large cage with conspecifics and plastic toys that are changed and rearranged daily. Most individuals that use addictive substances do not become addicted. Environmental enrichment mimics these individual differences in susceptibility. Therefore, the overall goal of this study is to identify novel therapeutic targets for treating neuropsychiatric

disorders using two discovery-based strategies. The first strategy to narrow the exciting leads is a convergent transcriptomic/proteomic analysis of mRNA and protein regulated by enrichment and cocaine. This strategy identifies AKT signaling as a promising pathway; therefore, we knocked down the downstream target of AKT, glycogen synthase kinase 3 (GSK3) beta, with a novel adeno-associated viral (AAV) vector in the nucleus accumbens shell (NAcSh) of rats and found increases in depression-like and cocaine taking behaviors. Additionally, GSK3 beta knockdown significantly reduced the activity of tonically active interneurons in the shell. The second discovery-based strategy is a convergent functional transcriptomics approach to examine the intersection of transcripts regulated by environmental enrichment, cocaine, and genes with enhanced expression in the NAcSh. NAcSh specific genes are identified through a topographic transcriptomic analysis with in situ hybridization from the Allen Mouse Brain Atlas. The second strategy identifies retinoic acid signaling as a potential underlying factor of enrichment and cocaine in the NAcSh. Therefore, we manipulated a gene with enhanced NAcSh expression in the retinoic acid signaling pathway, which was regulated by environmental enrichment, fatty acid binding protein 5 (FABP5), and found a decrease in cocaine taking behaviors. Thus, we identified novel targets through two discovery-based strategies investigating the underlying cellular mechanisms of environmental enrichment.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AAV-shCTRL	control vector
AAV-shFABP5	FABP5 knockdown vector
AAV-shGSK3β	GSK3β knockdown vector
ATF6	activating transcription factor 6
CART	cocaine and amphetamine regulated transcript
CREB	cAMP response element binding protein
CUD	cocaine use disorder
ERK	extracellular signal regulated kinase
FABP5	fatty acid binding protein 5
GABA	gamma-aminobutyric acid
GO	Gene Ontology
GOBP	Gene Ontology Biological Processes
GOCC	Gene Ontology Cellular Compartment
GOMF	Gene Ontology Molecular Functions
GSEA	Gene Set Enrichment Analysis
GSK3β	glycogen synthase kinase 3 beta
IPA	Ingenuity Pathway Analysis
IV	intravenous
MAPK	mitogen-activated protein kinase
MAPT	microtubule associated protein tau
MEF2	myocyte enhancer factor 2

MSN	medium spiny neuron
NAc	nucleus accumbens
NAcSh	nucleus accumbens shell
NES	normalized enrichment score
NFE2L2	nuclear factor, erthroid 2-like 2
NTN1	netrin 1
OCT1	octamer-binding transcription factor 1
PSEN1	presenilin 1
RA	retinoic acid
RICTOR	rapamycin-insensitive companion of mTOR
SA	self-administration
SMUG1	single-strand-selective monofunctional uracil-DNA glycosylase 1
STARD5	steroidogenic acute regulatory protein (StAR) related lipid transfer domain containing 5
STRA6	stimulated by retinoic acid 6
TANs	tonically active neurons
TFT	Transcription Factor Targets
VDR	vitamin D receptor
YY1	yin and yang 1 transcription factor

CELLULAR MECHANISMS OF ENVIRONMENTAL ENRICHMENT: NOVEL DISCOVERY-BASED STRATEGIES FOR TARGET IDENTIFICATION FOR NEUROPSYCHIATRIC DISORDERS

1. OVERVIEW

SUBSTANCE USE DISORDERS

Addiction is a brain disease characterized by compulsive drug use with a loss of control over drug taking that can interfere with daily activities (Koob and Le Moal, 2001). Substance use disorders (SUD) are highly prevalent, with approximately 1 in 10 individuals having suffered from an SUD in their lifetime (Conway et al., 2006). A decade ago, abuse of illicit drugs was estimated to cost the United States nearly $200 billion annually (Center, 2011). Current treatment options are largely limited to temporary rehabilitation programs and cognitive behavioral therapy, with varying success. Currently, cocaine use disorder (CUD) has no FDA approved pharmacotherapeutic; therefore, novel targets for pharmacotherapeutic development for CUD are required.

Substance use disorders are highly comorbid with mood disorders. Many individuals with cocaine use disorder will also suffer from anxiety (31.2%) or depression (35.7%) within their lifetime (Conway et al., 2006). Many individuals with anxiety or depression will also suffer from cocaine use disorder (~6%) (Conway et al., 2006). Individuals with comorbid substance use and mood disorders often show greater severity of symptoms and are less responsive to treatment (Swendsen and Merikangas, 2000).

Thus, it is critical to determine novel targets for substance use disorders with comorbid mood disorders.

High comorbidity rates suggest there is a shared disease etiology. Indeed, substance use disorders and mood disorders are characterized by dysregulation of the reward circuitry and are precipitated by a complex interplay between genetic and environmental factors (Russo and Nestler, 2013; Nestler, 2015; Manning et al., 2017). A better understanding of the reward circuitry is necessary to aid novel target identification for these disorders.

Reward circuitry

The function of the reward circuitry is to respond to "natural rewards" (e.g. food, sex) to ensure behaviors critical for survival. The reward circuitry is composed of multiple brain regions that are highly interconnected including the nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala, hippocampus, and prefrontal cortex (PFC). The VTA is a midbrain structure that sends dopaminergic projections to other regions in the reward circuitry such as the NAc, amygdala, hippocampus, and PFC. The nucleus accumbens, or ventral striatum, receives this dopaminergic input from the VTA, which is critical for reward salience and motivation (Robinson and Berridge, 1993; Ikemoto and Panksepp, 1999). The VTA and the hippocampus also send projections to the amygdala, which aids in reward-related memory formation. The prefrontal cortex exerts executive control over behavior through glutamatergic projections to the other reward-related areas (Koob and Volkow, 2010). These regions work in concert to identify and respond to rewarding stimuli.

Substances that are highly addictive enter the brain and stimulate the reward circuitry at substantially higher levels than natural rewards. Cocaine in particular causes high levels of extracellular dopamine in the nucleus accumbens by blocking reuptake, resulting in euphoria or intense feelings of pleasure described as a "high". Continued use

of cocaine causes long lasting alterations in gene expression, synaptic plasticity, and even global structural alterations in the brain (Nestler, 2005; Hyman et al., 2006; Ma et al., 2009).

The nucleus accumbens is particularly important in the development of substance use disorders. Dopaminergic neurons from the VTA synapse onto GABAergic medium spiny neurons (MSNs) in the NAc, which are the most common cell type and the only output of the NAc. Dopamine release in this mesolimbic circuit is rewarding, contributing not only to initial responses to rewarding stimuli, but dysregulation of this circuit may also contribute to the development of addiction (Nestler, 2001). NAc MSNs are divided into two subtypes based on dopamine receptor expression. D1-type MSNs express D1 and D5 dopamine receptors while D2-type MSNs express D2, D3, and D4 dopamine receptors. These two populations of MSNs differentially modulate behavioral responses to cocaine (Lobo et al., 2010). Individual differences in dopamine receptor expression in the NAc may contribute to individual differences in susceptibility to substance use disorders (Dalley et al., 2007). In addition to dopaminergic inputs from the VTA, the NAc also receives glutamatergic inputs from the amygdala, hippocampus, and PFC. Altered glutamatergic signaling to the nucleus accumbens is associated with the transition from drug use and abuse to addiction (Kalivas, 2004; Kalivas and Volkow, 2005). Activity of NAc MSNs in response to cocaine is modulated by NAc interneuron populations, particularly tonically active neurons (TANs), which are typically cholinergic (Lenz and Lobo, 2013).

The nucleus accumbens is functionally divided into an outer shell region and an inner core region. The nucleus accumbens shell (NAcSh) is critical for the initial responses to unconditioned stimuli, such as addictive substances, as well as repeated drug-taking behaviors (Meredith et al., 2008; Malenka et al., 2009). Cocaine preferentially stimulates the release of dopamine in the shell over the core (Pontieri et al., 1995) and rats will self-administer infusions of cocaine into the shell (McKinzie et al.,

1999). Therefore, this dissertation focuses on the outer region of the nucleus accumbens, the shell region (NAcSh).

ANXIETY AND DEPRESSION

Like substance use disorders, anxiety and depression are disorders of the reward circuitry with the nucleus accumbens playing a significant role in the development and maintenance of these disorders (Russo and Nestler, 2013). In this dissertation, mood disorders refer to anxiety and depression specifically, although other disorders such as bipolar disorder are also classified as mood disorders. Anxiety is characterized by excessive worrying that is difficult to manage while depression is characterized by depressed mood and loss of interest (i.e. loss of pleasure). Studies in humans have shown a role of the NAc in anxiety and depression as deep brain stimulation of the NAc in humans can temporarily reduce the symptoms of both anxiety and depression (Sturm et al., 2003; Bewernick et al., 2010). Rodent studies have also shown a role of the NAc in anxiety- and depression-like behaviors. Modulation of the aforementioned NAc tonically active neurons, which are typically cholinergic and modulate NAc MSNs, can increase depression-like behavior in rodents (Warner-Schmidt et al., 2012). Some antidepressant drugs have even shown anticholinergic activity (Berton et al., 2006; Zarate et al., 2013). Investigation of the nucleus accumbens is therefore critical for a better understanding of mood disorders.

Current treatment options for anxiety and depression include cognitive behavioral therapy as well as several pharmacotherapeutics. Antidepressant drugs, particularly selective serotonin reuptake inhibitors (SSRIs), and benzodiazepines are commonly used treatments for anxiety (Duman and Duman, 2005). Antidepressants are often ineffective (remission rate 30-40%) (Krishnan and Nestler, 2011), especially in individuals with mild to moderate depression, which constitutes a large majority of patients (Penn and Tracy, 2012). Not only are pharmacotherapeutics ineffective in many patients, but in order to

determine effectiveness, patients often must endure a time consuming process of successively higher doses of a drug and, when it has no effect on their symptoms, a slow weaning process in order to try a different drug. Further, many pharmacotherapeutics take several weeks to begin to see any alterations in the symptoms of these disorders. The original antidepressant drugs were discovered by chance several decades ago and pharmacotherapeutics since then have been largely based on the original drugs, which exert effects by increasing the neurotransmitters dopamine, serotonin, or norepinephrine (Nestler et al., 2002; Penn and Tracy, 2012). Thus, the identification of promising novel targets is necessary to develop improved pharmacotherapeutics for these psychiatric disorders.

ENVIRONMENTAL ENRICHMENT: ANIMAL MODEL OF RESILIENCE TO DEPRESSION AND ADDICTION

In order to better understand anxiety, depression, and addiction, as well as identify novel targets, this dissertation is a preclinical investigation of the susceptibility and resilience to these disorders. The focus is on the reward circuitry, specifically the nucleus accumbens, and an animal model that shows resilience to depression-like and addiction-related behaviors, called environmental enrichment.

Environmental enrichment is a non-drug, non-surgical, non-genetic manipulation that produces a protective depression phenotype and a protective addiction phenotype. Environmental enrichment involves housing the animals in different environments. In the enriched condition (EC), animals are reared in a large cage (approximately 70 cm x 70 cm x 70 cm) with other animals the same age (i.e. conspecifics) and several plastic objects that are changed and rearranged daily (Figure 1.1). The EC animals are compared to the isolated condition (IC), where the animals are singly housed in a standard sized cage without additional objects. By utilizing this differential housing of animals, we can examine rats that are resilient to depression and addiction (enriched condition) and rats

that are susceptible to depression and addiction (isolated condition). Because enrichment is not a genetic or drug-induced manipulation, but rather the result of differential housing, it as an ideal animal model to study the molecular mechanisms of depression and substance use disorders.

Three hallmark symptoms of depression in humans are social withdrawal, behavioral despair, and anhedonia (i.e. the inability to feel pleasure) (Brenes Saenz et al., 2006; Brenes et al., 2008; Green et al., 2010). Enriched rats showed a protective depression phenotype in three depression-like behavior tests that model these aspects of human depression, including the social contact test, the forced swim test, and the sucrose preference test (Green et al., 2010). Enriched rats spent more time grooming each other in the social contact test, the spent more time swimming during the forced swim test, and they consumed more sucrose water (1%) in the sucrose preference test as compared to isolated rats (Green et al., 2010).

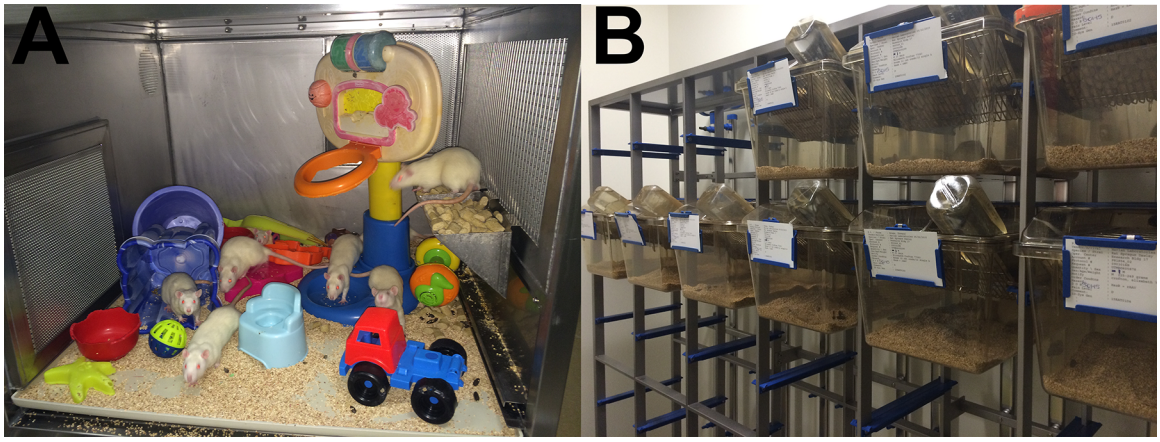

Figure 1.1 Environmental enrichment and isolation housing conditions
A. Enriched condition (EC). B. Isolated condition (IC).

Enriched rats also show a protective addiction phenotype. Figure 1.2 is adapted from Green et al., 2010 and illustrates the protective addiction phenotype conferred by environmental enrichment in the cocaine self-administration paradigm. Enriched rats took longer to acquire cocaine self-administration at low dose (0.2 mg/kg/infusion, Panel A)

and high dose cocaine (0.5 mg/kg/infusion, Panel B). Figure 1.2 shows a rightward shift in the ascending limb of the dose response function in EC rats compared to IC rats in terms of the number of responses made per dose (Panel C) as well as a decrease in intake in EC rats in terms of the amount of cocaine taken per dose (Panel D). EC rats extinguished the lever pressing behavior more quickly than IC rats when cocaine was not available (Panel E) and EC rats made fewer responses after a high unit reinstatement dose of cocaine (Panel F).

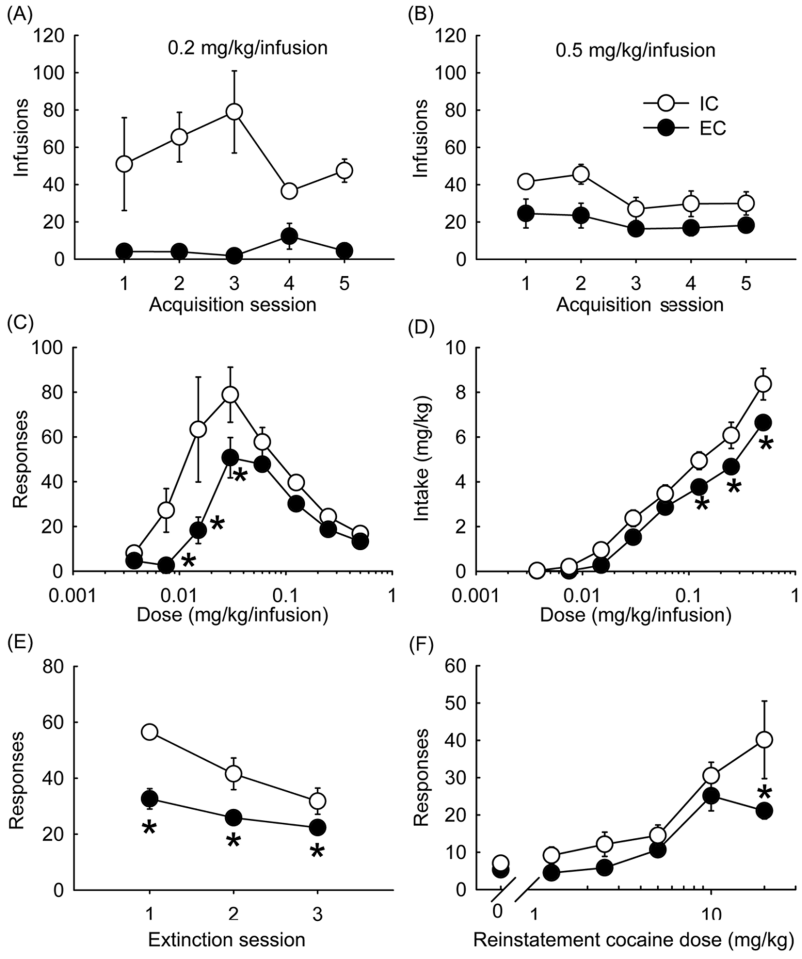

Figure 1.2 Protective addiction behavioral phenotype of environmental enrichment

Acquisition of cocaine self-administration at 0.2 mg/kg/infusion dose (Panel A) or 0.5 mg/kg/infusion dose (Panel B). (C) Dose-response function based on responses, (D) dose response of intake at each dose, (E) extinction, and (F) reinstatement. Open circles are isolated animals and filled circles are enriched animals. Adapted from Green et al 2010, with permission from Elsevier.

Enriched rats show a protective addiction phenotype not only for cocaine but also amphetamine, ethanol, and methylphenidate (Bardo et al., 2001a; Green et al., 2002; Green et al., 2010; Alvers et al., 2012). Enriched rats show altered sensitivity to many drugs of abuse with disparate mechanisms of action, suggesting that the rearing environment has altered their reward circuitry in such a way that the reinforcing effects of multiple drugs are affected. Investigation of the underlying mechanisms of environmental enrichment therefore will yield novel targets critical for the resilience to depression and addiction.

Inoculation stress hypothesis of environmental enrichment

There is some debate on how environmental enrichment produces these protective behavioral phenotypes. Chapter 2 posits that the enriched rats are exposed to mild stress that inoculates them against future more severe stressors, including drugs of abuse. This mild stress in the enriched rats leads to resilience, while the lack of adaptive stress exposure in the isolated rats leads to increased susceptibility. It is not clear the specific cellular or molecular alterations that cause these phenotypes, although some genes have been identified, such as DeltaFosB (Zhang et al., 2014) and CREB (cAMP response element binding protein, (Green et al., 2010)). Further investigation is required to fully understand the underlying mechanisms of environmental enrichment.

Most individuals that use addictive substances do not become addicted. Environmental enrichment mimics these individual differences in susceptibility to psychiatric disorders seen in humans. Therefore, investigation of the underlying mechanisms of environmental enrichment will lead to a better understanding of psychiatric disorders as well as novel targets for pharmacotherapeutic development.

RATIONALE FOR STUDY

There is no FDA approved pharmacotherapeutic for CUD and individuals with comorbid mood disorders and substance use disorders are often symptomatically the most severe as well as highly treatment-resistant. Thus, the overall purpose of this dissertation is to identify and validate novel targets for therapeutic development for comorbid mood disorders and cocaine substance use disorder. **We hypothesize that novel targets for neuropsychiatric disorders can be identified through discovery-based investigation of the underlying cellular mechanisms of environmental enrichment, a rodent model of resilience to depression and addiction.**

Historically in neuropsychiatric disease research, genes of interest are investigated separately at the individual target level. This type of reductionist approach is limited to known targets of interest and therefore not designed to illuminate novel pathways or targets. Neuropsychiatric disorders are complex disorders resulting from multiple factors and thus the underlying mechanisms must be examined at the genome-wide level without *a priori* defined targets to enhance novel target identification. Technological advancements in robust and reproducible large-scale quantification of transcripts and proteins allow for such discovery-based genome-wide experiments. However, difficulty remains in interpreting the results of genome-wide analyses as well as determining the biological relevance of the resultant data. Additionally, there is a paucity of studies integrating multiple genome-wide analyses, especially for neuropsychiatric disorders. Integration across different analyses at multiple levels could provide a better understanding of the etiology of these disorders and provide novel targets for further investigation.

Environmental enrichment provides a unique animal model to investigate the underlying mechanisms of the resilience to these disorders. Thus, we aim to identify

novel targets for comorbid psychiatric disorders with the following discovery-based integrative strategies accompanied by the validation of promising targets.

Strategy 1. Convergent transcriptomic/proteomic analysis of environmental enrichment and cocaine.

Strategy 2. Convergent transcriptomic analysis of genes with enhanced expression in the nucleus accumbens shell (topographic transcriptomics), environmental enrichment, and cocaine.

Large-scale genome-wide analyses

Large-scale genome-wide analyses have been developed in order to better understand a disease or phenotype. These analyses have been termed "-omics" studies from the shared suffix of the various approaches (i.e. genomics, transcriptomics, proteomics, metabolomics). Here we focus on transcriptomic (mRNA) and proteomic (protein) analyses in order to investigate the underlying cellular mechanisms of environmental enrichment. These discovery-based strategies will generate novel hypotheses, which will then be tested.

Recent advances in machinery, sample preparation, and software for the statistical analysis of the resulting omics data has made these types of studies more affordable, more widely available, and more accessible to biomedical researchers. However, there remains a need for effective strategies to go beyond differential expression to investigation of pathway level relationships and aid in interpretation of the biological significance. The usage of computational methods to understand complex biological data is termed bioinformatics.

TRANSCRIPTOMICS

The most pivotal technological advancement in transcriptomics, or RNA level analysis, is next generation RNA sequencing (NGS). NGS replaces the time consuming

and labor intensive methods of Northern blots and Sanger sequencing (Ari and Arikan, 2016). The Human Genome Project resulted in the development of an improved method of sequencing involving sequencing hundreds of short fragments (or reads) at a time and mapping each fragment to a reference genome or reference sequence (Ari and Arikan, 2016). The number of reads mapped to each gene allows for accurate quantification of transcripts. NGS requires minimal labor and can sequence RNA from any source.

We used the well-accepted and widely used NGS platform from Illumina (Illumina Hi-seq) for the sequencing and quantification of enriched or isolated rats that self-administered cocaine or saline (Zhang et al., 2016a) to investigate the underlying mechanisms of enrichment. The Illumina Hi-seq system uses fluorescently labeled nucleotides in order to determine the sequence, base by base. The Illumina Hi-seq is particularly adept at efficient quantification of the whole transcriptome with near-perfect accuracy (99.9%), and in our case, sequenced 50 base pair reads at a depth of 100 million reads (i.e. the average number of times a base in the sample is sequenced) (Ari and Arikan, 2016; Zhang et al., 2016a). Thus, we are able to identify and quantify mRNA from thousands of genes from one side of the nucleus accumbens of rats without prior identification of these targets.

PROTEOMICS

Advances have also been made in the large-scale quantification of proteins. Methodology has improved from gel-based approaches to separation of protein mixtures with high performance liquid chromatography paired with tandem mass spectrometry (nanoLC-MS/MS). Gel-based approaches such as two-dimensional gel electrophoresis followed by mass spectrometry are labor intensive and limited to only regulated proteins with relatively high abundance (Gygi et al., 2000; Crofton et al., 2017a). Mass spectrometry was originally reserved for the analysis of chemicals, but has been repurposed for the identification of peptides in complex mixtures through the ability to

ionize and spray peptides into the mass spectrometer. In general, proteins are digested into smaller peptides with trypsin, separated by liquid chromatography, converted to ions and sprayed into two mass spectrometers in parallel that measure the mass to charge ratio and the relative abundance of each fragment. We used nanoLC-MS/MS to identify and quantify proteins from the other side of the nucleus accumbens from the same rats as the transcriptomic analysis (Lichti et al., 2014).

Discovery-based bioinformatic analysis

Bioinformatic analysis of omics datasets goes beyond differential expression of individual targets and focuses on pathway level relationships between targets. Bioinformatic analysis can utilize overrepresentation statistics to determine if there are more genes regulated in a pathway than expected by chance. Pathway level investigations are somewhat resistant to type I error ("false positives"). This type of analysis assumes that false positives will be randomly distributed throughout the dataset and that biological regulation will not be randomly distributed, but rather occurs in a coordinated fashion. Bioinformatic software aids in the analysis as well as the visualization of large omics datasets. Thus, it is particularly useful for examining complex, multidimensional biological processes such as the underlying mechanism of individual differences in susceptibility to neuropsychiatric disorders.

Strategy 1: Convergent transcriptomic/proteomic analysis of environmental enrichment and cocaine

The first strategy employed for novel target identification is a convergent transcriptomic/proteomic analysis of environmental enrichment. One side of the accumbens from enriched or isolated rats that self-administered cocaine or saline was previously used to identify differentially regulated transcripts (Zhang et al., 2016a) while the other side from the same rats was used to identify differentially regulated proteins

(Lichti et al., 2014). Individually, the previous core transcriptomic and proteomic analyses provide one-dimensional information. This secondary convergent analysis compares the transcriptomic and proteomic data and was previously published by Zhang and Crofton et al., 2016 (co-first authors). This convergent approach will provide two-dimensional complementary information as well as cross-validation of each core analysis. We hypothesize that an integrative transcriptomic/proteomic analysis of the nucleus accumbens of environmentally enriched or isolated rats will identify a promising target for further investigation.

It is assumed that since proteins are translated from mRNA, alterations in mRNA and protein levels will correlate with one another. Some reports of transcriptomic and proteomic analyses indicate that even in bacteria, mRNA and protein do not show a strong correlation (Maier et al., 2009; Zhang et al., 2010). One question of interest for this strategy is whether mRNA and protein expression changes correspond to one another. Additionally, this convergent analysis is unique as the mRNA and protein were taken from the same rats (Lichti et al., 2014; Zhang et al., 2016a). Low correspondence at the individual target level indicates the targets may be regulated, not just by transcription and translation, but also by post-translational modifications, etc. Correspondence at the pathway level indicates the pathway may be important for the resilience to depression-like and addiction-related behaviors.

As our strategy employs a convergent approach to the data by comparing mRNA and protein regulation, we analyzed the data in multiple commonly used ways by utilizing multiple software programs. Using different bioinformatic software programs increases the confidence in the pathways or targets identified by more than one program. In order to examine the similarities and differences in the pathway level relationships between mRNAs and proteins regulated by environmental enrichment, we used three bioinformatic software programs, Ingenuity Pathway Analysis (IPA) (Qiagen, www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/), Perseus (Max

Planck Institute, www.perseus-framework.org), and Gene Set Enrichment Analysis (GSEA) (Broad Institute, http://software.broadinstitute.org/gsea/index.jsp).

IPA uses a manually curated database of information from the published literature of interactions between genes, shared functions, upstream effectors including drugs and microRNAs, pathway level relationships, etc. However, IPA is not freely available and is therefore constrained to usage by researchers with a license.

A customary way to analyze omics data is through gene ontology. In general, gene ontology refers to the collection of information known about genes, as well as established relationships between genes. We utilized two software programs, Perseus and Gene Set Enrichment Analysis (GSEA), for gene ontology analysis of mRNAs and proteins. Perseus and GSEA examine genes related by their molecular function, cellular compartment, biological process, or shared transcription factor regulation.

Ingenuity Pathway Analysis

IPA determines whether a query list of genes are overrepresented in curated gene sets from a "Knowledge Base" of interactions between genes, chemicals, and molecules from peer-reviewed publications. IPA calculates a p-value for the curated gene sets with Fisher's Exact Test, which takes into account the number of genes in each gene set or pathway. A z-score for each pathway is also calculated if fold change or expression values are available for the gene list. A z-score is a prediction of whether the pathway is activated (z-score > 2) or inhibited (z-score < 2) based on data from published literature. The main output of IPA includes Canonical Pathways, Diseases and Biological Functions, and Upstream Regulators. Canonical pathways are gene sets organized by a shared cellular pathway such as *CREB signaling*. Diseases and Biological Functions are gene sets organized by demonstrated associations between these genes like a shared disease or biological function. The upstream regulator analysis predicts drugs, molecules, microRNAs, or other genes that are upstream of the genes in the query data set. IPA is a

powerful tool as it includes statistical analyses, standard and custom visual representations of the data, as well as a well-maintained database of published interactions between genes. However, one limitation of IPA is that the curated knowledge base is not specific to the nervous system, but rather contains relationships from a variety of published articles.

Perseus

Perseus is a freely available software package with a multitude of functionalities (Tyanova et al., 2016). We utilized Perseus to compare the transcriptomic and proteomic data sets as Perseus has a functionality called 2D annotation enrichment (Cox and Mann, 2012), which can compare the quantitative data from two disparate datasets, regardless if it is protein or transcript data. This analysis uses a "two-dimensional generalization of the nonparametric two-sample test" and controls for multiple comparisons (false discovery rate) (Cox and Mann, 2012). The mRNA and protein data were analyzed for correspondence in three large gene ontology categories (Biological Processes (GOBP), Molecular Functions (GOMF), and Cellular Compartment (GOCC)). Thus, each gene ontology category can be defined by the regulation found at the mRNA and protein levels.

Gene Set Enrichment Analysis

GSEA is also a freely available software package. However, GSEA determines statistical significance in a different manner compared to Perseus and IPA. GSEA ranks all of the genes in a gene set based on change in expression level between two phenotypes (Subramanian et al., 2005). GSEA determines whether genes in a pathway or gene set are significantly upregulated or downregulated in one phenotype compared to another by calculating an enrichment score. An enrichment score (ES), or running sum statistic (Kolmogorov-Smirnov statistic), is calculated by ranking all of the genes in a pathway by differential expression level, then determining overrepresentation at the top

(positive ES) or the bottom (negative ES) of the list. The enrichment score is normalized to adjust for multiple comparisons (gene set size), which results in a normalized enrichment score (NES). The pathways and gene sets used by GSEA are categorized by a variety of functions or processes from the Molecular Signatures Database (MSigDB). Our analysis focused on transcription factor target gene sets and curated gene sets. Transcription factors regulate a variety of downstream targets and have previously been identified as involved in the protective phenotypes of environmental enrichment (Green et al., 2010; Pavlovsky et al., 2013; Zhang et al., 2014). GSEA includes various curated gene sets from different sources including the Reactome Pathway Database, containing classic signaling pathways with interaction data from the published literature.

Strategy 2: Convergent transcriptomic analysis of environmental enrichment, cocaine, and genes with enhanced expression in the NAcSh

The second strategy employed for novel target identification is a convergent transcriptomic analysis of three transcriptomic data sets. The three data sets are the mRNAs in the nucleus accumbens regulated by the environmental enrichment main effect, the mRNAs in the nucleus accumbens regulated by the cocaine main effect, and a list of genes identified via a topographic analysis of the nucleus accumbens shell.

Evolution influences gene expression and ensures that genes are expressed largely only where they are needed. The nucleus accumbens has been studied extensively as an integral part of the reward circuitry and alterations of this region can promote the development of psychiatric disorders including anxiety, depression, and addiction (Hope et al., 1992; Dong et al., 2006; Green et al., 2006; Nestler and Carlezon, 2006; Green et al., 2008; Green et al., 2010; Zhang et al., 2014). Genes with enhanced expression in the nucleus accumbens shell may therefore be critical for reward-related behaviors and may influence the development of mood disorders and/or substance use disorders. In fact, some genes with demonstrated roles in reward related behaviors do show enhanced

expression in the shell (e.g. cocaine and amphetamine regulated transcript (CART), serotonin receptor 2C (HTR2C), and activating transcription factor 3 (ATF3)) (Douglass et al., 1995; Douglass and Daoud, 1996; McMahon and Cunningham, 1999; Green et al., 2008). We hypothesized that a discovery-based approach will identify other genes with NAcSh enhanced expression critical for cocaine taking and seeking behaviors. We utilized the Allen Mouse Brain Atlas, a freely available resource with genome-wide *in situ* hybridization data for the entire mouse brain (mouse.brain-map.org), to probe for genes with specific expression in the nucleus accumbens shell over nearby regions, particularly the rest of the striatum.

Additionally, we hypothesized that an evaluation of the intersection of environmental enrichment, cocaine, and the NAcSh enhanced genes can identify a novel pathway critical for the resilience to depression-like and addiction-related behaviors. We used Ingenuity Pathway Analysis to investigate the pathways shared between the resulting list of genes with enhanced NAcSh expression with the enrichment and cocaine mRNA data and to narrow the many leads to the most promising pathway. A signaling pathway with many genes with enhanced expression in the NAcSh as well as regulated by enrichment and cocaine may prove to be an effective target for pharmacotherapeutics for comorbid cocaine use disorder and mood disorders.

TARGET VALIDATION

The overall hypothesis of this dissertation is that novel targets for psychiatric disorders can be identified through discovery-based investigation of a resilient rodent model and the nucleus accumbens. Above we outline the two discovery-based strategies utilized to narrow the many exciting leads to the most promising targets. The targets identified through these two strategies must be validated *in vivo* to provide causal evidence for their role in reward-related behaviors as well as validation of the discovery-based strategies that identify the targets. We therefore utilized RNA interference via

novel adeno-associated viral vectors to knockdown expression of the identified targets in the nucleus accumbens shell of rats and examined reward-related behaviors.

In vivo knockdown of targets with viral vector-mediated RNA interference

RNA interference, which is triggered by the presence of double-stranded RNA, is a post-transcriptional mechanism that cells use to silence genes as well as regulating mRNA levels, regulating translation, and fighting viral infections (Fire et al., 1998). RNA interference can be used to knock down a specific mRNA of interest through expression of a short hairpin RNA (shRNA) sequence (Hannon, 2002; Reynolds et al., 2004a). An efficient method of introducing an shRNA sequence targeting a gene of interest is by packaging the shRNA sequence with a mammalian promoter into a viral vector (Hommel et al., 2003). The viral vector transfects the cell and causes the cell to express the shRNA sequence at a high level. When expressed, the shRNA forms a hairpin, or a segment of double stranded RNA connected by a loop of single stranded RNA. The shRNA is expressed using the cells machinery and is likewise processed by an RNase named Dicer into just the double stranded RNA (dsRNA) segment. Dicer incorporates the dsRNA into a complex of proteins called the RISC (RNA-induced silencing complex) (Hannon, 2002). Inside the complex of proteins, the dsRNA is separated and the accompanying strand is degraded. Generally, the strand used for silencing is the strand that is less tightly bound at the 5' end (Khvorova et al., 2003; Schwarz et al., 2003; Reynolds et al., 2004a). For identification of the correct mRNA, the antisense strand must be loaded into the RISC. The remaining RNA strand then guides the RISC in identification of mRNAs for degradation by the Argonaute protein in the RISC (Hannon, 2002). Exogenous shRNA introduced with a viral vector is a very efficient way of achieving sustained depletion of the normal expression of a gene of interest (Hommel et al., 2003; Reynolds et al., 2004a). RNA interference through exogenous shRNA is utilized in this dissertation to knockdown expression of identified targets, as the knockdown is restricted to cells that endogenously

express the target genes. The shRNA will only affect cells that express the shRNA as the mRNA for the target protein hast o be already expressed in the cell.

Adeno-associated viral (AAV) vector

The delivery system used here to introduce the shRNA sequence into the cell is the adeno-associated viral vector. The adeno-associated virus is a small replication-deficient virus that transfects dividing and non-dividing mammalian cells (Daya and Berns, 2008). AAV does not cause any known diseases in humans, making it a relatively safe vector for use in the laboratory (Daly, 2004; Daya and Berns, 2008). Additionally, genetic material can be packaged into recombinant AAV without a replicating virus. AAV is packaged by transfection of HEK293-derived cells (AAV-293) with a plasmid with inverted terminal repeats (ITR) from the wild type virus along with the shRNA (or a gene of interest for overexpression), a plasmid that expresses the replication and capsid genes, and a helper plasmid that takes the place of a helper virus, providing genes necessary to create the viral particles (Agilent Technologies Manual, www.agilent.com/library/usermanuals/Public/240071.pdf). AAV particles are produced in the AAV-293 cells and are loaded with the shRNA, then purified from the cells. Thus the shRNA plasmid is packaged into the AAV viral particles to form the viral vector.

AAV is an excellent vector not only for its safety and relatively low toxicity, but it can be packaged at very high viral titer ($>10^{12}$particles/mL), which is necessary when injecting a small volume into a specific brain region. The AAV vectors are injected directly into the nucleus accumbens shell with a stereotaxic instrument using bregma as a guide. Thus, AAV-mediated shRNA transfer allows for knockdown of target genes specifically in the nucleus accumbens shell as well as manipulation of genes in adult rodents without causing developmental disruptions.

Additionally, AAV vectors *in vivo* express at high levels after two weeks and maintain expression for months, even years (Vassalli et al., 2003; Aschauer et al., 2013).

This is ideal for self-administration experiments, which can last several weeks to months. However, because the envelope of AAV is small, it can only hold constructs that are similar in size to the wild type AAV genome (~4.7kb), which would be a hindrance when overexpressing a gene (Daly, 2004). The shRNA construct used in this dissertation is 70bp, making it ideal for AAV-mediated expression. Thus the adeno-associated viral vector, with high titer, transfection of dividing and non-dividing cells, and long-lasting expression, is ideal for manipulation of targets in a target brain region.

The plasmids used in this dissertation contain AAV ITRs, shRNA under a U6 promoter, green fluorescent protein (GFP) under a CMV (cytomegalovirus) promoter, and are packaged into AAV2 serotype (Figure 1.3). The U6 promoter is an RNA

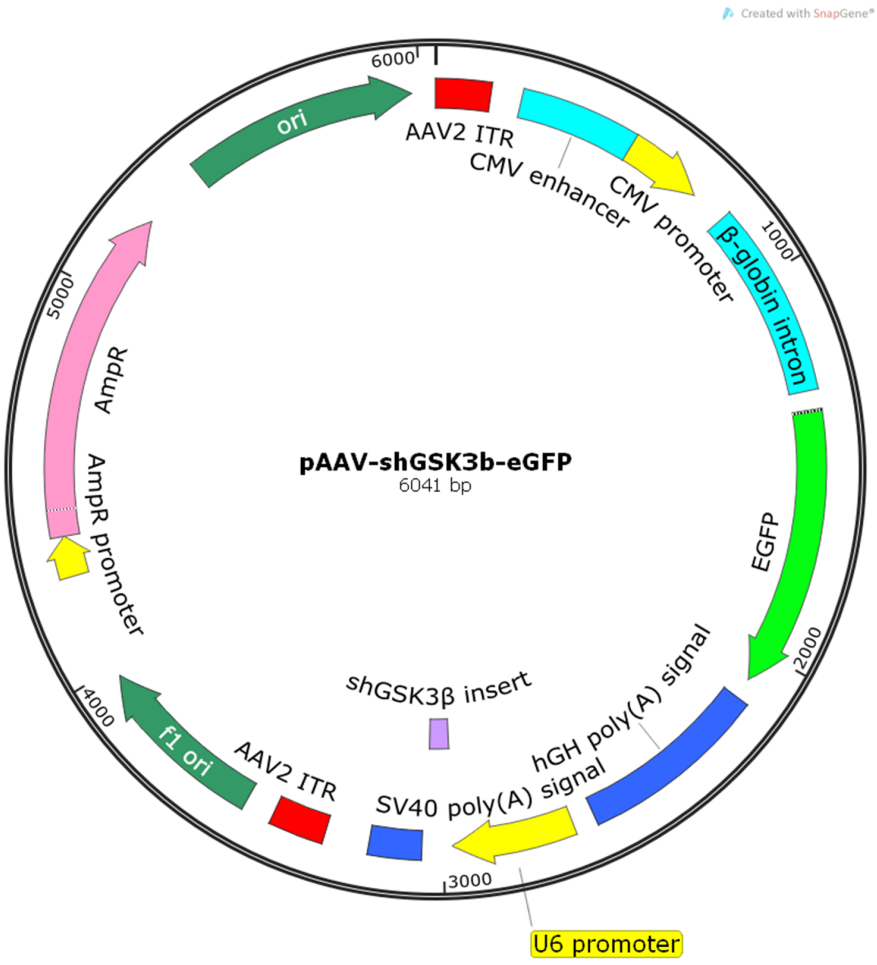

Figure 1.3 Plasmid map for AAV-shGSK3β

polymerase III promoter and is particularly effective for shRNA expression (Reynolds et al., 2004a). The CMV promoter is an RNA polymerase II promoter, ensuring that the expression of the shRNA is not affected by the expression of GFP (Hommel et al., 2003). GFP expression along with the shRNA allows for simplified visualization of the location of viral vector transfection. GFP can even be visualized with royal blue fluorescent flashlight and yellow barrier filter goggles (Nightsea, Bedford, MA) (Anastasio et al., 2014; Crofton et al., 2017b).

Serotypes of AAV have various tropisms for specific cell types as well as direction of transfection. The AAV2 serotype is utilized because it contains specific proteins in the capsid that cause it to transfect neurons *in vivo* (Summerford and Samulski, 1998). We manipulated the identified targets *in vivo* with novel AAV2 shRNA vectors and measured alterations in behavior.

Models to study rodent behavior

After identification of promising targets with two discovery-based bioinformatic strategies, in order to validate these targets, we developed novel AAV vectors to knockdown expression of these targets *in vivo*. We hypothesized that knocking down the expression of the identified targets in the nucleus accumbens shell of rats would produce alterations in reward related behaviors. Of particular interest is whether the behavior of the knockdown animals mimicked that of resilient rats (enriched) or susceptible rats (isolated). Multiple animal models have previously been validated for investigation of anxiety-like, depression-like, cocaine-taking, and cocaine-seeking behaviors (Koob and Volkow, 2010; Nestler and Hyman, 2010; Krishnan and Nestler, 2011).

Anxiety-like behavior

Anxiety-like behavioral tests were developed by validation with anxiolytic drugs (Pellow and File, 1986; Miyata et al., 1992; Merali et al., 2003). In order to provide

confidence in the anxiety-like phenotype of the animals, a battery of anxiety-like tests that assess anxiety-like behavior under different conditions are employed in this dissertation. Anxiety-like tests used include elevated plus maze, sucrose neophobia, and cold-stress induced defecation. These anxiety-like behavioral tests are utilized as they do not involve exposure to severe stress (Green et al., 2010; Crofton et al., 2015) and they can be assessed as part of a screen of behavioral tests in the same rats (Green et al., 2010; Zhang et al., 2014).

The most commonly used anxiety-like behavior test is the elevated plus maze (EPM) (Pellow and File, 1986). Rats are placed on an apparatus with four raised arms. Two arms have walls while the other two arms and the junction between the arms do not have walls. Rodents naturally have an inclination to explore a novel area but are also wary of open areas. A control animal spends a majority of the time in the closed arms while also venturing out onto the ends of the open arms. Generally, animals are placed onto the EPM for 5 minutes and time spent in each arm and the junction area is recorded either with video recording and hand scoring or a computer monitoring activity with beam breaks. Anxiolytic drugs increase the amount of time spent on the open arms. An animal that spends more time in the closed arms is showing increased anxiety-like behavior.

Another model of anxiety-like behavior is the sucrose neophobia test (Merali et al., 2003). Like the EPM, rats naturally are inclined to explore but are also cautious of novel foods. Once the novel food is deemed safe, the rats will consume the food. The sucrose neophobia test involves exposing the rats to a 1% sucrose solution in water and quantifying the amount of sucrose they drink within a brief period (Green et al., 2008; Green et al., 2010). Rats that consume more sucrose are showing anxiolytic-like behavior.

The final anxiety-like model employed in this dissertation is the cold stress-induced defecation test (Miyata et al., 1992; Green et al., 2010). A natural response to

stress or fear is defecation. The amount of fecal boli can therefore be considered an indirect measure of the amount of anxiety an animal is experiencing. The cold stress-induced defecation test involves exposing animals to a mild stressor (a cage placed on ice) for a brief period of time (less than half an hour) and quantifying the amount of fecal boli produced (Green et al., 2010; Crofton et al., 2017b). Rats that produce more fecal boli are showing increased anxiety-like behavior.

Depression-like behavior

The depression-like behavior tests used are designed to model two different aspects of depression, anhedonia and social withdrawal (Nestler et al., 2002; Nestler and Hyman, 2010). The first, anhedonia, or the inability to feel pleasure, is indirectly measured with the sucrose preference test (Willner, 2005). Rats are given two bottles, one with water, and one with the 1% sucrose solution (Green et al., 2010; Zhang et al., 2014; Crofton et al., 2017b). The amount of sucrose compared to water can be quantified by comparing the amount of sucrose and the amount of water consumed. The rats are first exposed to the sucrose solution in the sucrose neophobia test so that the preference test measures their willingness to drink the more palatable solution. Rats that drink less sucrose compared to controls are showing increased depression-like behavior. The second depression-like behavior test is the social contact test, which models social withdrawal (Green et al., 2010; Zhang et al., 2014; Crofton et al., 2017b). Pair housed rats are separated for 24 hours, then reunited in a novel environment and the amount of time spend grooming one another is measured. A pair of rats that spend more time grooming each other compared to controls are showing decreased depression-like behavior. Both the sucrose preference test and the social contact test are utilized in order to provide confidence in the results as well as these tests limit stress exposure (Crofton et al., 2015). Prior exposure to severe stress can increase acquisition of cocaine self-administration (Haney et al., 1995; Covington and Miczek, 2005). Thus, these mild stress

depression-like tests can be assessed as part of a battery of behavioral tests prior to cocaine self-administration.

Cocaine self-administration

Cocaine self-administration is the addiction model with the highest face validity compared to human substance use disorders and most closely resembles human drug taking behavior (Haney and Spealman, 2008; O'Connor et al., 2011). Drug self-administration allows for investigation of aspects of human addiction (Koob and Volkow, 2010). In this paradigm, an animal is placed in a two-lever operant chamber with cue lights above each lever and an infusion pump that can deliver cocaine to a jugular vein catheter implanted subcutaneously (Crofton et al., 2017b). Operant self-administration can be analyzed with a variety of phases including acquisition, maintenance, extinction, reinstatement, which are akin to phases of human drug abuse (Arroyo et al., 1998). The cocaine self-administration experiments in this dissertation were limited to reinforcement on a fixed ratio 1 (FR1) schedule, or one infusion per lever press, as environmental enrichment can cause alterations in cognitive ability (Diamond et al., 1964; Renner and Rosenzweig, 1987; Rosenzweig and Bennett, 1996). Acquisition is the first daily sessions where the rats have access to the drug and models initial drug use. The maintenance phase is a period of stable responding for repeated sessions at the same dose. The extinction phase is non-reinforced responding in order to decrease the rate of responses during periods of no drug. The reinstatement phase is when the animal is stimulated to start responding again after extinction by receiving a priming dose of the drug either intraperitoneal or through the IV catheter. Reinstatement can be elicited with a variety of stimuli including stress or exposure to cue lights or even returning the animal to the operant chamber after several days without the drug (i.e. abstinence)(Shaham et al., 2003). The dose response function examines the animal's sensitivity to the drug (Ahmed and Koob, 2005).

OVERVIEW

The following chapters outline a novel hypothesis of environmental enrichment (Chapter 2) as well as two discovery-based bioinformatic strategies to discover novel targets through analysis of the protective depression and addiction phenotypes of environmental enrichment (Chapters 3 and 5). Chapter 4 validates the target identified with strategy 1, AKT signaling, through *in vivo* knockdown of glycogen synthase kinase 3 beta (GSK3β). Chapter 5 validates the target identified with strategy 2, retinoic acid signaling, through *in vivo* knockdown of fatty acid binding protein 5 (FABP5). This dissertation therefore identifies and validates novel targets for comorbid cocaine substance use disorder and mood disorders through two discovery-based bioinformatic analyses of environmental enrichment and the nucleus accumbens.

2. INOCULATION STRESS HYPOTHESIS OF ENVIRONMENTAL ENRICHMENT[1]

Molecular analyses of neuropsychiatric disorders in humans are limited to non-invasive measurements such as blood samples and cerebral spinal fluid or post-mortem brain tissue. Animal models in contrast can be analyzed at the molecular level with a multitude of techniques as well as analyzed for behavioral differences. Animal models that closely resemble aspects of human disease would be ideal for genome-wide discovery-based analyses. This dissertation focuses on investigation of the animal model environmental enrichment, as enrichment is a non-drug, non-genetic, and non-surgical manipulation that results in protective depression and addiction phenotypes.

Environmental enrichment models individual differences in susceptibility to neuropsychiatric disorders, which is also seen in humans. Stress in humans can precipitate depression or relapse to drug use but adaptive stress exposure can decrease the risk of developing these disorders (Felitti et al., 1998; Khoshaba and Maddi, 1999; McEwen, 2000; Rutter, 2006; Lyons et al., 2009; Seery et al., 2013). This chapter provides a novel hypothesis that enriched rats are resilient to depression-like and addiction-related behaviors because of stress inoculation. The enriched environment constitutes constant mild stress exposure through novelty, social contact, and exercise, which protects against future stressors.

This chapter was peer-reviewed and published in the journal *Neuroscience & Biobehavioral Reviews* by Crofton et al.; it is reproduced here with permission[1].

[1] Reprinted from Neuroscience & Biobehavioral Reviews, 49, Elizabeth J. Crofton, Yafang Zhang, Thomas A. Green, Inoculation stress hypothesis of environmental enrichment, 19-31, 2015, with permission from Elsevier. doi: 10.1016/j.neubiorev.2014.11.017.

HISTORY OF ENVIRONMENTAL ENRICHMENT RESEARCH

The "nature vs. nurture" debate began in earnest during the Victorian period, championed by Sir Francis Galton, who was inspired by the works of his cousin Charles Darwin. At issue was whether a person's expressed traits are a product of heritability (i.e. nature) or by his/her own experiences (nurture). Galton, bolstered by Darwin's theories on heritability came down firmly on the side of "nature". The opposing "nurture" side of the debate was best defined centuries before by John Locke's borrowed term "tabula rasa" (i.e. blank slate). The "nurture" side of the argument was further strengthened in the early 1900s by John Watson's theories on behaviorism. As science evolved (particularly the advent of genetics), the "nature vs. nurture" debate evolved into a "genes vs. environment" debate, respectively. The battle raged on as scientists on both sides of the argument produced irrefutable evidence for their view. Eventually, scientists realized that both arguments were correct— that a person's expressed phenotype was due to an interaction of genes with environment. Thus, the Gene/Environment Interaction Theory was born. In a basic sense, the environment controls (to some degree) how genes are expressed. Thus, gene transcription is where the proverbial "rubber hits the road" and seems to play a significant role in the protective phenotypes produced by environmental enrichment (Green et al., 2010; Lobo et al., 2013; Zhang et al., 2014) which are described below in the beneficial effects of environmental enrichment. The beginning of modern environmental enrichment research is mostly attributed to Rosenzweig, Renner, Bennett, Diamond, and colleagues. This group used the environmental enrichment paradigm to show convincingly that the adult brain still exhibits plasticity and that, just like muscles, brains get stronger with greater use. Rats reared in an enriched condition (EC) have a thicker cortex, more dendritic arborization, and greater cognitive abilities than rats reared in an isolated condition (IC) (Diamond et al., 1964; Renner and Rosenzweig, 1987; Rosenzweig and Bennett, 1996). Following these early experiments, many others have

used environmental enrichment and found it to be a useful animal model in a variety of fields, particularly because it is a non-drug and non-surgical manipulation. In parallel with Rosenzweig and colleagues, Harry Harlow was finalizing the ideas for his seminal work on the importance of maternal and social enrichment in rhesus monkeys (Harlow, 1958). Harlow designed inanimate wire and cloth "surrogate" mothers to show that maternal contact is enriching to baby macaques beyond merely providing food. Although Harlow's early work was oriented to the positive effects of maternal enrichment (i.e. affection), his later work shifted perspective to focus on the isolation aspect (i.e. lack of enrichment) rather than the enrichment itself (Harlow and Suomi, 1971).

WHAT IS ENVIRONMENTAL ENRICHMENT?

Environmental enrichment is complex and there are numerous ways to provide enrichment. There is a lack of consistency in protocols for enrichment between different laboratories, but the most common procedure in rats involves rearing the subjects in a large cage with novel objects and social contact with conspecifics for at least 30 days beginning immediately after weaning. The objects are replaced and rearranged daily to maximize novelty. This arrangement provides three key facets of enrichment: novelty, social contact, and exercise. It has been shown in rats that all three aspects are rewarding (Bevins and Bardo, 1999; Bardo and Bevins, 2000; Belke, 2000) and all three release dopamine in the nucleus accumbens (Louilot et al., 1986; Rebec et al., 1997; Greenwood et al., 2011). Thus, it can be said that environmental enrichment is a compound manipulation that provides a daily workout for the dopamine system. Indeed, when the novel objects are replaced each day, the rats display a burst of exploratory activity lasting approximately 30 min that is beyond anything seen with locomotor stimulants like cocaine or amphetamine. Additionally, there is a second burst of exploratory/play behavior that occurs at the onset of the dark cycle, the beginning of the rats' normal period of high activity. Although environmental conditions have a dramatic impact on the

behavior of animals, these differing protocols for enriching rats often produce conflicting results. Parameters such as age of the animal, degree of enrichment, duration of enrichment, species, and sex can each affect the results of an experiment. The lack of consistency in protocols likely stems from a lack of consensus regarding the definition of what indeed constitutes "environmental enrichment". Some might define enrichment based on environmental complexity—that a more complex environment is more enriching; however, environmental complexity alone is not the whole story. Environmental enrichment, by most definitions, should exert a positive influence on the organism, setting enrichment apart from overtly stressful events that have a negative impact on the organism. Thus, enrichment must provide an overall benefit to the organism. Further confusion in the field also arises from the fact that some researchers compare EC rats only to pair-housed social condition (SC) rats or compare only IC with SC rats (see below for discussion of the appropriate control for enrichment). However, without discounting or dismissing the views of others studying environmental enrichment using different protocols, this chapter outlines a theory that the mild daily stresses of the enriched lifestyle are adaptive and inoculate rats to produce protective preclinical phenotypes for addiction and depression.

What are the beneficial effects of environmental enrichment?

As mentioned above, environmental enrichment contains three basic components: novelty, exercise, and social contact. Animals are group-housed in a large cage equipped with children's plastic toys, which are replaced and rearranged every day. In order to study the "preventive" effect of environmental enrichment, rats are usually raised in the enriched condition before exposure to drugs (in the case of addiction research) or stress (in the case of depression research). Environmental enrichment attenuates the reinforcing effects of addictive drugs and produces an antidepressant-like effect (Brenes Saenz et al., 2006; Brenes et al., 2008; Laviola et al., 2008; Green et al., 2010). In addition,

environmental enrichment can be studied as a "treatment" model, in which rats are assigned to either an isolated or enriched condition after they are exposed to drugs or stress, which has also been shown to produce adaptive consequences (Grimm et al., 2008; Solinas et al., 2008; Thiel et al., 2010). Below is an overview of the beneficial effects of environmental enrichment. To maintain focus, this chapter is predominantly centered on rodent research, although environmental enrichment has been studied in other species with success (Harlow, 1958; Harlow and Suomi, 1971; Solinas et al., 2010; Nader et al., 2012b).

The protective addiction phenotype

Rats reared in an enriched condition exhibit lower basal locomotor activity than rats in the isolated condition, making interpretation of drug-stimulated locomotor data challenging. Despite this, the available evidence is fairly clear that EC rats show greater locomotor sensitivity to psychostimulants such as amphetamine and cocaine (Bowling et al., 1993; Bowling and Bardo, 1994; Bardo et al., 1999; Smith et al., 2009) while at the same time showing reduced sensitization to repeated exposure (Bardo et al., 1995; Smith et al., 1997; Green et al., 2003). In the conditioned place preference (CPP) paradigm, a paradigm more relevant to addiction, Bowling and Bardo, and then Green and colleagues reported that enrichment produces enhanced CPP to both amphetamine and cocaine in rats (Bowling and Bardo, 1994; Green et al., 2010). These results lead to the hypothesis that enrichment increases the risk for addiction; however, in the paradigm with the greatest face validity for addiction, the intravenous drug self-administration paradigm, the reinforcing effects of amphetamine, cocaine, ethanol, and methylphenidate are decreased by enrichment and the rats self-administer less of these drugs (Bardo et al., 2001a; Green et al., 2002; Stairs et al., 2006; Deehan et al., 2007; Green et al., 2010; Alvers et al., 2012; Gill et al., 2013). Enrichment also alters drug taking in treatment models. Exposure to enrichment after exposure to cocaine reduces locomotor activity, eliminates cocaine

CPP and reduces cocaine-induced reinstatement of CPP, and decreases cocaine-seeking behavior during extinction and cue-elicited reinstatement (Solinas et al., 2008; Thiel et al., 2009; Ranaldi et al., 2011; Chauvet et al., 2012). Although cue-elicited reinstatement is reduced in the treatment model, enrichment does not alter cocaine-primed reinstatement, suggesting that enrichment reduces the salience of drug-associated environmental cues which could lead to an effective therapy for craving elicited by drug cues in humans (Thiel et al., 2009).

The protective antidepressant phenotype

Along with the protective addiction phenotype, environmental enrichment also produces a protective antidepressant-like phenotype. In humans, three of the hallmark symptoms of depression are anhedonia, social withdrawal, and behavioral despair. Our prior research and others found that, compared to IC rats, EC rats consume more sucrose in a sucrose preference test, indicating decreased anhedonia-like behavior; longer grooming time in the social contact test, suggesting decreased social withdrawal; and greater mobility time in the forced swim test, suggesting reduced "behavioral despair" (Brenes Saenz et al., 2006; Brenes et al., 2008; Green et al., 2010).

Anxiety

Similar to addiction- and depression-like behavior, multiple labs have demonstrated reduced anxiety-like behavior resulting from environmental enrichment. For example, EC rats display lower basal locomotor activity, yet increased distance traveled in the center of the arena in the open field test, indicating an anxiolytic effect (Urakawa et al., 2013). In addition, enriched rats and mice were found to spend more time in the open arms in the elevated plus maze (EPM), and showed lower amounts of defensive burying and less defensive behavior when in close proximity to a predator, also suggesting reduced anxiety (Roy et al., 2001; Friske and Gammie, 2005; Leal-Galicia et

al., 2007). Enriched mice show reduced anxiety in response to social defeat stress, an effect that was abolished by lesioning the infralimbic region of the prefrontal cortex prior to environmental enrichment exposure (Lehmann and Herkenham, 2011), suggesting a role of the prefrontal cortex in anxiety and environmental enrichment. However, not all are convinced that enrichment is anxiolytic. Our own research demonstrated greater sucrose neophobia in EC rats and more fecal boli in the cold-stress defecation test, both suggesting increased anxiety (Green et al., 2010). In addition, latency to ejaculation has been used as a measure of anxiety (Wallace et al., 2009), and EC rats exhibit increased latency to ejaculate, again suggesting increased anxiety (Urakawa et al., 2014). It is noteworthy that the last three tests (sucrose neophobia, cold-stress defecation, and latency to ejaculation) are not a function of exploratory activity in a novel environment. Because environmental enrichment involves extended exposure to novel environments whereas IC rats have very little to no experience in novel environments, this confounds the results of behavioral tests involving exploration of a novel environment. In addition, environmentally enriched rats show less social withdrawal than isolated rats, which may confound the results of social defeat tests on enriched animals (Green et al., 2010). Taken as a whole, the benefits of enrichment on anxiety-like behavior are not as clear as other areas, but further analysis of anxiety-like behavior in enriched animals is an interesting avenue for future experimentation.

Other disorders

Environmental enrichment has also been shown to have beneficial effects in rodent models of neurodegenerative diseases. For example, in a mouse model of Huntington's disease, enrichment delays the onset and slows disease progression by minimizing the loss of cerebral volume and by rescuing protein deficits (van Dellen et al., 2000; Hockly et al., 2002; Spires et al., 2004). Enrichment can also prevent neurodegeneration in C57BL/6 mice caused by a neurotoxin that causes Parkinson's

disease-like symptoms in humans by regulating expression of the dopamine transporter (DAT) (Bezard et al., 2003). In addition, an increasing number of studies reported the beneficial effect of environmental enrichment in improving learning and memory in behavioral and molecular aspects of Alzheimer's disease (Jankowsky et al., 2003; Levi et al., 2003; Jankowsky et al., 2005; Wolf et al., 2006; Bouet et al., 2011). A study on Tg2576 mice, a model of Alzheimer's, found that environmental enrichment counteracts the deleterious effects of chronic unpredictable stress in Alzheimer's disease progression (Jeong et al., 2011). Further, a recent proteomic study found that the microtubule-associated protein tau was upregulated in EC rats compared to IC rats (Fan et al., 2013b; Fan et al., 2013a). That paper and two other proteomics papers also identified huntingtin, presenilin 1, tau, and amyloid precursor protein as major upstream regulators for environmental enrichment (Fan et al., 2013b; Fan et al., 2013a; Lichti et al., 2014). These results warrant further investigation of neurodegenerative disorders using the environmental enrichment paradigm.

Species differences

Although environmental enrichment has been studied extensively in rats, other species have garnered considerably less attention, save for the work of Harlow in monkeys described above (Harlow, 1958; Harlow and Suomi, 1971). However, the available evidence from the primate literature suggests that enrichment is a protective factor for stress, as well as for addiction-related behavior (Harlow, 1958; Harlow and Suomi, 1971; Kozorovitskiy et al., 2005; Nader et al., 2012a). There are, however, some important rat/mouse differences in the effects of environmental enrichment. For example, rats show the unusual phenotype where environmental enrichment increases responsiveness to stimulants such as cocaine or amphetamine in locomotor, CPP, and neurochemical studies, while these same rats show decreased drug self-administration (Bowling et al., 1993; Bowling and Bardo, 1994; Bardo et al., 1999; Bardo et al., 2001b;

Green et al., 2002; Green et al., 2010); mice, on the other hand, show decreased sensitivity to stimulants after environmental enrichment (Solinas et al., 2009). Regardless, environmental enrichment produces a net benefit in addiction related behavior in both species (Solinas et al., 2010).

WHAT IS INOCULATION STRESS?

As described above, the environmental enrichment paradigm is a non-drug, non-surgical preclinical animal model useful for studying various psychiatric and neurological conditions. Environmental enrichment produces protective phenotypes in addiction and depression models, which are robust and replicable. The major question is how environmental enrichment can produce such robust individual differences in a wide range of behaviors associated with addiction, depression and anxiety and beneficial effects even for animal models of neurodegenerative diseases. Our hypothesis is that enriched rats undergo inoculation stress. In short, chronic very mild stress from living in a complex environment and interacting non-aggressively with conspecifics inoculates enriched rats against subsequent stressors and/or drugs of abuse. Inoculation stress, described previously in human studies, is a process of developing resilience to future stressful events by first being exposed to mildly stressful experiences early in life (Dienstbier, 1989; Khoshaba and Maddi, 1999; Parker et al., 2004; Fox et al., 2006; Rutter, 2006; Meichenbaum, 2007; Lyons et al., 2009). Exposure to stress or adversity that toughens an individual is protective, much like a vaccination that exposes an individual to a non-harmful version of a disease in order to develop immunity to that illness for the future (Rutter, 2006; Lyons et al., 2009). For example, adults who are exposed to work stress as adolescents have fewer negative health effects from work-related stress as adults (Mortimer and Staff, 2004). Unlike a vaccine, however, inoculation stress does not protect only against a single disease, but toughens the individual in general and prepares them to cope with a variety of stressors later in life. For example, individuals that have

successfully coped with some adverse events in adolescence have overall better mental health and are better able to cope with serious illness, spousal loss, or a major accident as adults (Khoshaba and Maddi, 1999; Lyons et al., 2009; Seery et al., 2013). An important distinction to make is that inoculation stress is not simply exposure to any stress early in life, but rather having positive and adaptive responses to mild stressors is critical. Severe stress early in life often causes the individual to be more vulnerable to stress later in life whereas mild stress exposure with an adaptive response can protect the individual (Khoshaba and Maddi, 1999; Parker and Maestripieri, 2011). Stress that is inoculating conditions the individual, and provides specific coping strategies for exposure to future stressors, ultimately leading to resilience. Stress inoculation has also been described in non-human primates and rodents. Squirrel monkeys and rodents exposed to a manageable stress early in life are less stress-reactive in the future (Lyons et al., 2009; Lyons et al., 2010). In particular, environmental enrichment in adolescence is able to provide protection from the deleterious effects of subsequent stressors (Larsson et al., 2002; Parker et al., 2004; Fox et al., 2006; Lyons et al., 2009), providing the impetus for the hypothesis that environmental enrichment is a chronic mild stress environment leading to stress inoculation.

ALLOSTASIS AND ALLOSTATIC LOAD

In trying to make sense of how mild/moderate stressors are adaptive yet severe stressors are maladaptive, it helps to frame the picture with respect to allostasis and allostatic load. For the purposes of this dissertation, the term "allostasis" will be used in its broadest sense, disregarding the narrow application of this term only to energy regulation (McEwen and Wingfield, 2010). Thus, allostasis is defined as the process of returning a dynamic system to its stable set point after a challenge to that system. In a conceptual sense, homeostasis is maintaining stability in a non-dynamic system while allostasis is regulating stability of a dynamic system. The genome is chock full of

allostatic mechanisms for maintaining stability in hundreds of dynamic systems. However, maintaining stability of a dynamic system is not without cost to the organism. This cost to the organism is termed "allostatic load" and can be paid in a currency as diverse as energy usage, ion concentrations (e.g. neuronal activity), or protein turnover. Regulatory systems have evolved to deal with allostatic load to a certain extent, but most systems are susceptible to "allostatic overload" if the demands on the system outpace the allostatic capacity. At that point, the system fails to maintain stability and pathology develops. In the arena of psychiatric conditions, allostatic overload is evident when severe stressors surpass the allostatic capacity of the person and induce pathological conditions such as post-traumatic stress disorder, major depression, addiction, and anxiety disorders (among others). One thing that differentiates allostasis from homeostasis is that allostasis, by virtue of regulating a dynamic system, can predict future allostatic load and adjust its capacity accordingly in anticipation of that future load. For example, forcing the average person to run 12 miles will result in allostatic overload of many systems (energy, oxygen, joints, muscles) whereas the seasoned runner's body has predicted the possibility of a 12 mile run and adjusted the allostatic capacity of these systems to match the high load. As a result, instead of being pathogenic, subsequent stressors can even be motivating to the individual, allowing them to thrive in adverse conditions. Thus, individual responses to the same stressful event can be highly variable, and the resulting effect on the individual depends on that individual's allostatic capacity to handle stress. Allostatic overload of stress response systems can lead to psychiatric disorders, but how can inoculation stress or exposure to mild stress become protective against developing psychiatric conditions? Unfortunately, stress has garnered a negative connotation, making experiments on stress with humans and animal models sometimes ambiguous. In 1976, Hans Selye defined stress as "the nonspecific response of the body to any demand made upon it" (Selye, 1976). The term "stress" does not inherently specify whether the stimulus is adaptive (i.e. inoculation stress) or maladaptive (i.e. allostatic

overload), but even in Selye's own work it was often assumed that "stress" meant maladaptive stress. However, whether a stressor is positive or negative chiefly depends on the allostatic capacity of the individual. Most individuals exposed to stress are resilient to psychiatric conditions but those that are susceptible have a lower allostatic capacity for stress. Inoculation stress causes the organism to predict future stress and increase the allostatic capacity of the individual accordingly, providing resilience to subsequent stressors that might otherwise produce psychiatric conditions. Thus, environmental enrichment is hypothesized to increase allostatic load capacity from the repeated exposure to very mild stresses, inoculating against subsequent stressors.

Since allostatic capacity for stress is associated with psychiatric disorders, it is useful to assess allostatic load capacity for stress in rodents. There is a wide range of stressors from which to choose to preclinically examine allostatic load capacity. Mildly stressful stimuli include brief periods of restraint stress, mild footshock, brief exposure to temperature changes as in the cold stress-induced defecation paradigm, and handling by an experimenter (Gärtner et al., 1980; Gregus et al., 2005; Green et al., 2010; Rabasa et al., 2011a). Some severe stressors are long periods of immersion in cold water, long periods of food or water deprivation, and intense foot-shock (Willner, 1997; Rabasa et al., 2011a; Rabasa et al., 2011b). One very severe stressor is social defeat by an aggressive conspecific (Koolhaas et al., 1996; Muller et al., 2000; Covington and Miczek, 2005). It is important to note that individual stressors can be considered mild or severe depending on duration and intensity. For example, brief maternal separation in rats is adaptive whereas lengthy maternal separation leads to allostatic overload and susceptibility in models of psychiatric conditions (Lyons et al., 2009; Sih, 2011). Thus, footshock, maternal separation, physical restraint, and temperature changes can be considered mild stressors or severe stressors. Predictability also seems important; the so-called chronic unpredictable mild stress paradigm (CMS), despite the label of "mild", can be a strong enough insult to induce allostatic over-load and prevent inoculation (see

below). Therefore, evaluating the response of the rodent to various stressors can aid in gauging the allostatic capacity of the rodent (i.e. whether they are inoculated against future stressors).

STRESS, ADDICTION AND DEPRESSION

As mentioned in the previous section, severe stress can lead to various psychiatric disorders; therefore, understanding the consequences of adaptive and maladaptive stress is translationally relevant. Stress is not only implicated in conditions such as post-traumatic stress disorder, major depression, and anxiety disorders, but also drug use disorders. Severe stress in adolescence (such as being abused as a child) is associated with higher risk for alcoholism, substance abuse, depression, suicide attempts, obesity, and poorer general health (Felitti et al., 1998; McEwen, 2000). Evidence suggests there is overlap in the underlying mechanisms for mental health disorders and drug use disorders (Levin et al., 2008; Worley et al., 2012), and psychological stress may be the common link. The environmental enrichment paradigm produces robust protective phenotypes for depression-like and drug abuse behaviors. Therefore, our hypothesis is not only does maladaptive stress increase the likelihood of psychological disorders and drug abuse, but also the protective effects of environmental enrichment may be due to adaptive responses to stress. Regarding stress and depression, stress is the leading factor for both the development of and relapse to major depression (Kendler et al., 1998; Kendler et al., 1999; Morris et al., 2010; Hardeveld et al., 2013). Stress in the workplace and major life events, such as the death of a spouse, can trigger depressive episodes and increase the risk of major depression (Heim et al., 1997; Tennant, 2001). Evidence suggests that alterations in the hypothalamo–pituitary–adrenocortical (HPA) axis, which is involved in coordinating the body's response to various stressors (i.e. managing allostatic load), are involved in depression. Cortisol is a glucocorticoid released by the adrenal cortex in response to environmental stimuli and individuals with higher cortisol reactivity to low

stress conditions had more depressive symptoms overtime than those with low cortisol reactivity (Morris et al., 2012). In order to study this phenomenon preclinically alterations in the hypothalamo–pituitary–adrenocortical axis should be evaluated.

Regarding addiction, stress is a leading factor contributing to relapse to drug use in humans (Pohorecky, 1991; Sinha et al., 1999; Sinha, 2001; Sinha et al., 2006), an effect modeled by self-administration studies in rats (Erb et al., 1996; Ahmed and Koob, 1997). Stress exposure in humans, particularly to a severe stressor, significantly increases cocaine craving during abstinence (Pohorecky, 1991). In addition to contributing to relapse, there is increasing preclinical evidence that stress contributes to the initial development of addiction (Piazza and Le Moal, 1998; Goeders, 2002; Covington and Miczek, 2005; Burke and Miczek, 2014). For humans, stress increases the likelihood that someone will start smoking along with increasing the risk of relapse to cigarette smoking (Bruijnzeel, 2012). Additionally, exposure to stress increases cocaine craving (Sinha et al., 1999) and more stress-induced cocaine craving increases the likelihood of relapse in cocaine-dependent individuals (Sinha et al., 2006). Beyond stress/addiction and stress/depression interactions, there is evidence linking addiction directly to depression (Levin et al., 2008; Worley et al., 2012). For example, individuals with major depression are more likely to smoke than the average person (Bruijnzeel, 2012). Major depression and substance use disorders are often comorbid in humans, and the symptoms are often more severe together than with only one disorder (Kessler et al., 2005; Pettinati et al., 2013). The rate of comorbidity of major depression with alcohol use disorders is 40.3%, and major depression with a drug use disorder is 17.2% (Pettinati et al., 2013). The high rate of comorbidity makes developing an effective treatment very complicated. If the mood disorder is solely substance-induced, controlling the drug use would solve both disorders, but if the mood disorder has some other etiology, then antidepressants may be required (Pettinati et al., 2013). Often treatment for individuals with these comorbid disorders is focused on one disorder or the other and disregards the fact that stress may

underlie both disorders. Depression and addiction phenotypes often go hand in hand not only in humans but also in rodents (Green et al., 2006; Green et al., 2008; Green et al., 2010; Pettinati et al., 2013). For example, overexpression of a dominant negative inhibitor of CREB (inducible cAMP early repressor/ICER or a mutant CREB/mCREB) or knockdown of CREB in the NAc produces an antidepressant-like phenotype and also decreases cocaine self-administration in a similar manner to environmental enrichment (Green et al., 2006; Green et al., 2010). Similarly, the transcription factor ΔFosB in the NAc is associated with stress and also cocaine-taking behavior (Vialou et al., 2010; Zhang et al., 2014). Thus, it is very difficult to tease apart addiction and depression phenotypes in humans and in rodent models. Therefore, analysis of an animal model that addresses both disorders simultaneously, such as environmental enrichment, is very valuable. Further, the close ties between depression and addiction are hypothesized to explain how inoculation to stress can affect drug-taking behavior. As a whole, the evidence linking stress, depression and addiction provides a plausible rationale for how the repeated mild stress of environmental enrichment can protect against addiction-related behavior.

DOES CORTISOL/CORTICOSTERONE EQUAL STRESS?

Because stress is linked with psychological disorders and drug dependence disorders, assessing stress in preclinical models is translationally relevant but can prove to be difficult without a good objective measure of stress in animals. Often, measurements of plasma cortisol, or the rodent equivalent corticosterone (CORT), have been used as a measure of stress in a variety of experiments. CORT is the primary glucocorticoid released by the adrenal cortex as a final product of the HPA axis. The HPA axis is activated in response to environmental stressors and activation of this system releases CORT. As a result, many scientists use CORT as a de facto indication of a subject's stress level. However, it is important to note that CORT does not equal stress.

The following paragraphs will argue: (1) CORT levels fluctuate throughout the day independent of stress, (2) rewarding stimuli induce CORT release, (3) CORT induction is attenuated with chronic stress, (4) CORT itself is reinforcing and (5) behavioral responses to CORT administration alone do not mimic responses to stress. In addition, there are several caveats when attempting to extrapolate emotional and behavioral state from plasma CORT levels, not the least of which is that the mere act of acquiring a blood sample to measure CORT can be stressful itself, especially with high frequency sampling (Abelson et al., 2005). Therefore, evaluating stress in environmentally enriched animals based on CORT levels has contributed to differing hypotheses on whether enrichment or isolation is inherently stressful because of inconsistent findings of corticosterone levels between enriched and isolated animals (Konkle et al., 2010). However, this is not to say that CORT is not important, or that corticosterone is not involved in the beneficial effects of environmental enrichment. In fact, CORT and the HPA axis may be involved in the inoculation stress of environmental enrichment (see Section 8 below for elaboration). However, measured CORT levels do not provide a complete picture of the adaptive or maladaptive nature of the stress responses of an animal.

CORT levels fluctuate throughout the day

CORT measurements taken at different times during the circadian cycle will vary because CORT has a characteristic circadian rhythm. Regardless of stress level, CORT generally peaks as rats awaken just prior to the dark cycle and is lowest at the beginning of the light cycle (Allen and Kendall, 1967; Butte et al., 1976). The dark cycle is the period where the animals are awake and highly active versus the light cycle when the animals are mostly sleeping. Thus, if one were to use CORT as a de facto measure of stress, the circadian rhythm of CORT will likely produce confounding results depending on when blood is collected.

Rewarding stimuli induce CORT

Phasic CORT release subsequent to an environmental stimulus is generally assumed to be an indication that the animal is in a negative emotional state and that the stimulus had a negative impact. For example, social defeat, where a test subject is physically dominated by a more aggressive conspecific, is a noxious stressor and causes release of CORT in the defeated male (Buwalda et al., 2012). However, assumptions of a negative state are not always true. For example, sexual activity, a positive and rewarding stimulus, releases similar amounts of CORT as social defeat (Buwalda et al., 2012). Another stimulus, exercise, which is regarded as positive and rewarding, can also cause an increase in cortisol in humans (Buono et al., 1986; Deinzer et al., 1997; Rojas Vega et al., 2006). Voluntary exercise can also increase circulating CORT in Sprague–Dawley rats (Fediuc et al., 2006). In addition, rewarding drugs, including cocaine, cause CORT release (Moldow and Fischman, 1987; Torres and Rivier, 1992). Both rewarding and noxious stimuli cause alterations in CORT; therefore, CORT levels alone cannot differentiate between negative and positive stimuli.

CORT induction is attenuated with chronic stress

If CORT is a de facto measure of stress, one would expect repeated stress to increase CORT. Multiple studies have shown, however, that induction of CORT after a mild/moderate stressor attenuates with repeated exposure to the stressor in rats (Natelson et al., 1988; De Boer et al., 1990; Magarinos and McEwen, 1995; Mizoguchi et al., 2001; Carter et al., 2004; Barnum et al., 2007; Rabasa et al., 2011a). In humans, cortisol release also habituates with repeated exposures to the same stressor although there are individual differences in cortisol responses to stress (Deinzer et al., 1997; Gerra et al., 2001). This evidence suggests that repeated stress increases the allostatic capacity to future stressors, which is a possible mechanism for the inoculation stress underlying environmental enrichment effects.

CORT is reinforcing

Additional evidence that CORT is not the same as maladaptive stress is demonstrated by the fact that CORT itself has reinforcing properties. Rats will intravenously (Piazza et al., 1991) and orally self-administer CORT (Deroche et al., 1993), causing release of dopamine in the nucleus accumbens (NAc) (Graf et al., 2013). CORT injections also potentiate amphetamine self-administration at medium and high doses of amphetamine (10 and 30 g/infusion) (Piazza et al., 1991). These experiments and others show that CORT is reinforcing at circulating levels similar to that released by mild stress (Piazza et al., 1991).

Responses to CORT differ from responses to stress

If CORT equals stress, then CORT administration should produce the same responses as stress. MacDougall and Howland found that rats injected with CORT versus rats exposed to 30 min of restraint stress (a mild stressor) had the same amount of circulating CORT, but only restrained rats showed changes in short- and long-term synaptic plasticity in the subiculum (MacDougall and Howland, 2013). Retana-Marquez et al. (1998) found that CORT injections were not able to mimic the behavioral effects of social defeat stress even at very high circulating plasma levels. Social defeat causes decreases in male sexual behavior and decreases in testosterone, whereas CORT injections do not (Retana-Marquez et al., 1998). Conversely, rats restrained for 6 h a day for 21 days did not show an increase in depression-like behavior in the forced swim test but rats injected with CORT did show an increase in depression-like behavior, suggesting that in some cases, elevated CORT can cause more maladaptive changes than mild restraint stress (Gregus et al., 2005). Thus, CORT and stress sometimes produce different behavioral effects, and CORT administration alone cannot reproduce the behavioral effects of stress; therefore, CORT does not equal stress. All told, it is clear that although stress usually releases CORT, the circulating level of CORT is not a direct measurement of stress level. Further, it is important to remember that animals in a chronically mild-

stress environment show attenuated rather than potentiated CORT induction to stress or drugs. If CORT is not an adequate measure of stress, how can we determine if enriched rats are actually more stressed than isolated rats?

ARE ENRICHED RATS REALLY STRESSED?

The inoculation stress hypothesis of environmental enrichment proposes that enriched rats are repeatedly stressed. However, at first sight, it is exceedingly difficult to make this case. Young male Sprague–Dawley rats (unlike mice) typically establish dominance hierarchies through play behavior and, as long as there are no female rats in the vicinity, typically do not feel the need to challenge these hierarchies over time. Thus, in this rat enrichment utopia, fighting is rare, food is plentiful, space is expansive, and rats get all of the novelty, social contact (rats are social creatures), and exercise they desire. Additionally, it has repeatedly been proposed that enrichment is the "functional opposite of stress" (Fox et al., 2006; Wright and Conrad, 2008; Solinas et al., 2010). If true, how can one make the case that enriched rats are chronically stressed?

Although there are multiple good lines of evidence suggesting enrichment produces the functional opposite of stress (Fox et al., 2006; Wright and Conrad, 2008; Solinas et al., 2010), none of the reports gives a possible explanation for how enrichment produces this effect. The inoculation stress hypothesis of environmental enrichment outlined here posits that enrichment is a chronic mildly stressful condition that induces neuronal and neuroendocrine plasticity leaving enriched rats more resistant (i.e. greater allostatic capacity) to overtly stressful stimuli. Environmental enrichment exposes animals to novelty, social contact, and exercise and multiple studies have found that these variables cause stress-like responses. Acute voluntary exercise induces the secretion of CORT (Fediuc et al., 2006) and exposure to novelty will induce secretion of both CORT and adrenocorticotropic hormone (ACTH) in rats (Hennessy et al., 1979; Piazza et al., 1991; Larsson et al., 2002; Ostrander et al., 2006). Finally, rats housed together with

conspecifics had higher circulating CORT levels than isolated rats suggesting that social contact also causes a stress-like response (Raz, 2013). However, in the absence of a truly reliable and objective measure of stress, one must rely on circumstantial evidence comparing the effects of enrichment with the effects of repeated mild stress (Table 1).

Table 2.1. Parallels between repeated mild stress and environmental enrichment

	Effect	**Repeated mild stress**	**Environmental enrichment**
Endocrine	Blunted CORT induction	Natelson et al., 1988, De Boer et al., 1990, Magarinos and McEwen 1995, Barnum et al., 2007, Rabasa et al., 2011b, Carter et al., 2004	Stairs et al., 2011, Stairs and Bardo 2009, Skwara et al., 2012
	Blunted ACTH induction	Gadek-Michalska and Bugajski, 2003	Skwara et al., 2012, Belz et al., 2003
	Enlarged adrenals	Swanson and van de Poll, 1983, Marti et al., 1993	Mlynarik et al., 2004, Moncek et al., 2004
	Blunted adrenaline release	Dobrakovova et al. 1990	Moncek et al., 2004
Neurobiology	ΔFosB accumulation in NAc	Perrotti et al., 2004, Lobo et al., 2013	Zhang et al., 2014, Lobo et al., 2013, Solinas et al., 2009
	Attenuated immediate-early gene induction in NAc	Shoji and Mizoguchi, 2010 (cFos), Green et al. 2008 (ATF3),	Zhang 2016 and Zhang et al. manuscript in preparation
Behavior	higher sensitivity to locomotor activating effects of amphetamine and cocaine	Deroche et al., 1992, Lepsch et al., 2005	Bowling et al., 1993, Bowling and Bardo 1994, Smith et al., 2009, Green et al., 2010
	decreased stimulant self-administration	Matthews et al., 1996, Moffett et al., 2006, *however, see:* Carroll and Meisch 1984, Piazza et al., 1990a, Goeders and Guerin 1994, Shaham and Stewart 1994, and Miczek and Mutschler 1996, Kosten et al., 2000	Bardo et al., 2001, Green et al., 2002, Stairs et al., 2006, Thiel et al., 2009, Green et al., 2010, Alvers et al., 2012, Puhl et al., 2012
	increased defecation under stress conditions	Jorge et al. 2010	Green et al., 2010
	decreased locomotor activity to novelty	Cruz et al., 2012	Bowling et al, 1993, Green et al., 2003, Green et al. 2010
Physiology	heart rate returns to baseline more quickly after stress	Carter et al., 2004, Chen and Herbert, 1995	Sharp et al., 2002
	lower body weight	Harris et al., 2004	Pena et al., 2009

As summarized in Table 1, there are numerous parallels between the effects of environmental enrichment and repeated mild stress, adding strength to the idea that enriched rats are chronically stressed. Due to the importance of stress as a contributing factor to depression and addiction (see above), the effects of chronic stress on the body have been studied in depth. Results of these studies have produced a clear picture of the endocrine, neurobiological, and behavioral sequelae of chronic stress in humans and in rat models. Environmentally enriched animals have also been assessed for the same endocrine, neurobiological, and behavioral effects. There is much evidence that repeated mild stress blunts CORT induction in response to subsequent stressors (Natelson et al., 1988; De Boer et al., 1990; Magarinos and McEwen, 1995; Carter et al., 2004; Barnum et al., 2007; Rabasa et al., 2011b) and environmental enrichment also results in blunted CORT induction to stress (Stairs and Bardo, 2009; Stairs et al., 2011; Skwara et al., 2012). Repeated exposure to severe stressors (i.e. those producing allostatic overload), such as social defeat stress, does not show reductions in CORT induction to subsequent stress (Barnum et al., 2007). Repeated stress also blunts stress-induced adrenocorticotropic hormone (ACTH) induction (Gadek-Michalska and Bugajski, 2003) and stress-induced release of adrenaline (Dobrakovova et al., 1990). Environmental enrichment also blunts ACTH induction (Belz et al., 2003; Skwara et al., 2012) and reduces stress-induced release of adrenaline (Moncek et al., 2004). In addition, enlarged adrenal glands have been found in environmentally enriched animals (Mlynarik et al., 2004; Moncek et al., 2004) and in animals exposed to repeated mild stress (Swanson and van de Poll, 1983; Marti et al., 1993). Environmental enrichment and repeated mild stress also both produce lower body weights (Harris et al., 2004; Pena et al., 2009) and following stress, the animal's heart rate returns to baseline more quickly (Chen and Herbert, 1995; Sharp et al., 2002; Carter et al., 2004). Not only do environmental enrichment and repeated mild stress show the same endocrine consequences, they also show concordant neurobiological consequences in the nucleus accumbens.

Environmental enrichment reduces induction of immediate early genes (IEGs) (Zhang et al.; Zhang, 2016), as does repeated stress (Alibhai et al., 2007; Green et al., 2008; Shoji and Mizoguchi, 2010). In contrast to other IEGs, environmental enrichment causes accumulation of basal ΔFosB protein in the nucleus accumbens (Solinas et al., 2009; Lobo et al., 2013; Zhang et al., 2014), which also occurs in repeatedly stressed animals (Perrotti et al., 2004; Lobo et al., 2013). Environmental enrichment also produces robust effects on behavior that show similarities to the behavior of repeatedly stressed animals. For example, enriched animals show increased defecation to a mild stressor (novel cage under cold conditions) (Green et al., 2010), and rats that were alternately restrained for 1 hour placed on a platform surrounded by water for 1 h a day for 5 days also show increased defecation (Jorge et al., 2010). Environmentally enriched animals are more sensitive to the locomotor activating effects of amphetamine (Bowling et al., 1993; Bowling and Bardo, 1994) and cocaine (Smith et al., 2009). Repeatedly stressed animals are also more sensitive to the locomotor activating effects of amphetamine and cocaine (Deroche et al., 1992; Lepsch et al., 2005). Despite being more sensitive to locomotor stimulants, environmental enrichment decreases spontaneous locomotor activity in response to a novel environment (Bowling et al., 1993; Green et al., 2003; Green et al., 2010). After mild stress, animals also show a similar decrease in locomotor activity when placed in a novel environment (Cruz et al., 2012). As described above and illustrated in Table 1, environmental enrichment and repeated mild stress have matching endocrine, physiological, neurobiological, and behavioral effects. Although this is circumstantial evidence, it supports the argument that environmentally enriched animals are in a state of chronic mild stress and this mild stress in adolescence inoculates against future stressors. The mild stress of environmental enrichment, however, is distinct from the stress of paradigms such as the chronic mild stress (CMS) paradigm or the chronic unpredictable stress paradigm in that environmental enrichment constitutes predictable stress that produces adaptive responses. The chronic mild stress paradigm typically involves

stressors such as food deprivation, water deprivation, brief exposure to another subject, lights on during the dark cycle, periods of titling the cage by 30 degrees, and long periods of wet bedding material which occur randomly throughout the week for several weeks (Willner et al., 1987; Willner et al., 1992; Murison and Hansen, 2001). The CMS procedure can reduce sucrose and saccharine preference after several weeks of this unpredictable stress exposure (Willner et al., 1987; Hatcher et al., 1997), an anhedonic-like effect that can be reversed by several weeks of treatment with a tricyclic antidepressant (Willner et al., 1987). However, the CMS paradigm does not typically have other depression-like effects, and under some conditions this paradigm can actually increase sucrose consumption, suggesting inconsistencies in the paradigm (Murison and Hansen, 2001). An inoculation stress interpretation of these inconsistent data would posit that the unpredictable nature of the CMS can elevate the "mild" stress to a level that induces allostatic overload and that the inconsistencies in anhedonic-like behavior are a function of degree. The CMS by Murison and Hansen (2001) may not have induced allostatic overload, thus producing an inoculating effect whereas the other two reports induced a more severe stress (Willner et al., 1987; Hatcher et al., 1997; Murison and Hansen, 2001). Indeed, Hatcher and Hansen reported only finding an anhedonic saccharin response when the CMS paradigm included food deprivation.

CORT as a possible mediator of the protective EC phenotype

The sections above argue that CORT is not the same as stress; however, that is not to say that CORT is irrelevant. In fact, CORT responses may contribute substantially to the environmental enrichment protective phenotypes. For example, the environmental enrichment protective addiction phenotype fits well with what is known of the influences of CORT on stimulant self-administration. As mentioned above, CORT itself can be self-administered by rats (Piazza et al., 1991; Deroche et al., 1993), but there also exists evidence that CORT plays a significant role in stimulant self-administration. For

example, higher CORT induction was associated with greater self-administration of low unit doses of cocaine regardless of whether the rats were stressed with contingent footshock, non-contingent footshock or no footshock (Goeders and Guerin, 1996b). Next, acquisition of cocaine self-administration can be completely blocked by bilateral adrenalectomy, partially reduced by pharmacological inhibition of corticosterone release by metyrapone in rats, and self-administration can be partially recovered by adding CORT to the drinking water (Goeders and Guerin, 1996a). Given that stimulants induce CORT release and blocking that release blocks self-administration, it is likely that the amount of CORT release from a stimulant determines the ability of low doses of that stimulant to engender or maintain self-administration. Blunted CORT release from stimulants in EC rats (Stairs and Bardo, 2009) could be the underlying molecular mechanism whereby inoculation stress produces the protective EC addiction phenotype. Further investigations are warranted to test this hypothesis.

Stress influences on self-administration

As discussed above, acute stress in humans is a major factor in relapse to addiction, so it is not surprising that acute stress in rodents produces reinstatement of cocaine seeking (Erb et al., 1996). However, the question at hand is how prior stress (i.e., not during or immediately before the session) affects subsequent stimulant self-administration. The logic in the above sections suggests that inoculation stress blunts CORT induction and that a blunted CORT response leads to less stimulant self-administration. Hence, one would predict that prior repeated mild stress (i.e. inoculation stress) would decrease drug self-administration. Two reports show that rats exposed to short-term maternal separation stress as pups later show reduced acquisition of cocaine self-administration at low unit doses (Matthews et al., 1996; Moffett et al., 2006). Despite these reports however, there are several reports showing that repeated stress increases stimulant self-administration (Carroll and Meisch, 1984; Piazza et al., 1990; Goeders and

Guerin, 1994; Shaham and Stewart, 1994; Miczek and Mutschler, 1996; Kosten et al., 2000). Multiple factors could account for this discrepancy. First, the stressors in some of these papers are severe stressors that would exceed the allostatic capacity of the rats. Second, it is possible (even likely) that inoculation stress is most pronounced in very young animals (as with maternal separation and environmental enrichment). Third, it is possible that many other factors affect self-administration and that one or more of these factors are at play in some of these experiments. Regardless, if the inoculation stress hypothesis of environmental enrichment is correct, one would predict that environmental enrichment would decrease stimulant self-administration, which is undoubtedly the case (Bardo et al., 2001a; Green et al., 2002; Stairs et al., 2006; Thiel et al., 2009; Green et al., 2010; Alvers et al., 2012; Puhl et al., 2012). In any case, the fact remains that EC rats have blunted CORT responses, blunted CORT responses are associated with less self-administration, and EC rats self-administer stimulants less readily than IC rats which supports the inoculation stress hypothesis.

WHAT IS THE BEST CONTROL FOR ENRICHMENT?

An important problem in the enrichment field is the difficulty of being able to compare results between labs because of inconsistencies in enrichment protocols and the use of different control groups. Environmental enrichment is a compound manipulation whereby rats are chronically exposed to novelty, social contact, and exercise. The most rigorous scientific approach would be to study each aspect individually and then in combination. For example, it would be nice to know the relative contributions of social contact vs. object novelty vs. exercise, and if the combination of these factors is redundant, additive or synergistic. However, it is not possible to fully separate these aspects because social interaction is a form of novelty and greatly increases activity (i.e. exercise). In addition, this approach would entail studying eight different conditions, and

we as scientists have an ethical obligation to reduce as much as possible the number of animals used in biomedical research (Council, 2011).

Additionally, as the number of conditions increases, there rapidly comes a point of diminishing returns where the cost (in money and time) of running an increasing number of conditions exceeds the small incremental benefit of the knowledge gained. Thus, the number of conditions must be limited. The fact that novelty, social contact and exercise all fall under the umbrella term "environmental enrichment" presupposes some commonality among the three constituents of enrichment. Indeed, each of these factors is rewarding to rats, and each releases dopamine in the nucleus accumbens, a critical brain region involved in stress, addiction, and depression (Louilot et al., 1986; Rebec et al., 1997; Greenwood et al., 2011). Accordingly, the many different conditions can now be reduced to as few as two: environmental enrichment and the appropriate control group. Identifying the appropriate control group for cocaine administration is easy—an injection with no cocaine. Logic would dictate that the appropriate control for environmental enrichment (composed of novelty, social contact and exercise) would be a group with the absence of novelty, social contact, and exercise (i.e. isolation). Because pair housing is a form of enrichment (Council, 2011), comparing EC to pair-housed "control" rats would be akin to comparing a 20 mg/kg cocaine group to a 10 mg/kg "control". The problem is that "standard" laboratory housing for rats is two per cage in most research laboratories, and as such, these pair-housed rats are viewed by many scientists to constitute the "normal", or control condition. From this viewpoint, instead of seeing a continuum of enrichment ranging from isolation to pair-housed to full enrichment, environmental enrichment is seen as one manipulation (compared to pair-housed rats) and environmental isolation is seen as a different kind of manipulation. At first sight, the case for isolation being a different kind of manipulation might seem to have merit. The case that many researchers make is that isolation itself is a stressor, and as such should not be used as a control. Indeed, many studies have shown that maternal separation (isolating

pups from dam) and neonatal isolation (isolating pups from dam and other pups) are significant stressors, evoking ultrasonic vocalizations and inducing CORT release in pups (Hennessy and Weinberg, 1990; Kuhn et al., 1990; Kehne et al., 1991; Levine et al., 1991; Viau et al., 1996; McCormick et al., 1998). In addition, acute isolation of group-housed rats also induces CORT, which is taken as a clear indication of stress (Takatsu-Coleman et al., 2013). Many researchers thus make the leap of considering isolation rearing a condition of chronic stress, citing large CORT induction from acute stress as evidence. However, the inoculation stress hypothesis states that enriched animals are in a state of chronic very mild stress. As described above, blunted CORT induction is a sign of chronic stress, which is seen in EC rats and chronic mildly stressed animals alike. Table 1 further illustrates the many other signs of chronic stress that EC rats show that IC rats do not show. It is important to make the distinction that exhibiting a greater response to a stressor is not the same as being chronically stressed—quite the opposite. The isolated animals essentially have a lack of daily stimulation (i.e. stress) and therefore show a greater response to stress than EC rats, which are constantly stimulated (i.e. stressed) and therefore show a lesser response to subsequent stress. Arguments frequently used in favor of pair-housed controls over isolated controls is that the IC group is not a "natural" condition, nor a "normal" condition, and has less "translational relevance" than pair-housed rats. Pair-housing rats in a laboratory is certainly not more translationally relevant than any other housing condition. The only place it is normal for two humans to be confined in the same small space is in prison. As for "normal", even wild rats are social animals and are found in groups rather than pairs that stick together constantly. Regardless, is a control group itself supposed to be "normal"? In the case of a pharmacological control group, would it be "normal" for a human to take an injection of saline rather than a drug? A control group should be the lack of a manipulation, but the pair-housed or social condition contains some of the variables of enrichment, namely social interaction and elements of exercise or play, and therefore is an intermediate level

of enrichment capable of producing some behavioral effects of full enrichment, but not all (Bardo et al., 2001a; Rosenzweig, 2003; Green et al., 2010; Zakharova et al., 2012). For as described above in the inoculation stress hypothesis, environmental enrichment is a combination of exercise, novelty, and social contact with conspecifics, all of which are mild stressors resulting in inoculation against future stressors. Neither social interaction, novelty, nor any other single variable can account for all of the effects of enrichment (Van Praag et al., 2000). However, the goal in using the environmental enrichment paradigm is not to tease apart the different aspects of enrichment or different gradations of enrichment but rather to determine differences between enrichment and the lack of enrichment to find the molecular determinants of the resilience to addiction and depression. Therefore, in our opinion in light of the inoculation stress hypothesis, the isolated condition is the correct control for enrichment and the inclusion of an intermediate group such as the social condition is unnecessary.

The inoculation stress hypothesis is an underappreciated framework for understanding many of the complexities of stress and an organism's response to that stress. This hypothesis also has utility as a scaffold for which to build other novel hypotheses concerning susceptibility and resistance to psychiatric conditions such as addiction and major depression. Accordingly, application of the inoculation stress hypothesis to the environmental enrichment paradigm helps clarify the nature of nurture (i.e. role of environment) and its contribution to the resilience to addiction- and depression-related behavior.

Additionally, research designed to investigate the underlying mechanism of this resilience could lead to the identification of novel targets for treatment or prevention of psychiatric disorders. Thus, subsequent chapters utilize discovery-based genome-wide analyses of environmentally enriched and isolated rats in order to identify novel targets as well as validate these targets *in vivo*.

3. STRATEGY 1: CONVERGENT TRANSCRIPTOMIC/PROTEOMIC ANALYSIS OF THE NUCLEUS ACCUMBENS OF RESILIENT RATS AND COCAINE[2]

Environmentally enriched rats show a resilient phenotype for depression and addiction while isolated rats show a susceptible phenotype. These phenotypes mimic individual differences in susceptibility and resilience to psychiatric disorders seen in humans as well as the high rates of comorbidity of mood disorders and substance use disorders. Therefore, investigation of the molecular mechanisms of environmental enrichment and isolation specifically in the nucleus accumbens will aid in discovery of novel therapeutic targets.

This chapter utilizes the first strategy employed in this dissertation of discovery-based integrative bioinformatics to identify novel targets for comorbid cocaine use disorder and mood disorders. The strategy is a convergent transcriptomic/proteomic analysis of the nucleus accumbens of environmental enrichment and cocaine. This is a secondary integrative and comparative analysis of previous transcriptomic (Zhang et al., 2016a) and proteomic (Lichti et al., 2014) core analyses. In the core transcriptomic study, high-throughput RNA sequencing was used to quantify more than 14,000 transcripts from one side of the nucleus accumbens of rats that were either reared in an enriched condition or isolated condition and self-administered cocaine or saline. The other side of the nucleus accumbens from these same rats was utilized to identify and quantify 1,900

[2] Reprinted from Neuroscience, 339, Yafang Zhang, Elizabeth J. Crofton, Xiuzhen Fan, Dingge Li, Fanping Kong, Mala Sinha, Bruce A. Luxon, Heidi M. Spratt, Cheryl F. Lichti, Thomas A. Green, Convergent transcriptomics and proteomics of environmental enrichment and cocaine identifies novel therapeutic strategies for addiction, 254-266, 2016, with permission from Elsevier. doi: 10.1016/j.neuroscience.2016.09.051.

proteins with high-performance liquid chromatography with tandem mass spectrometry in the core proteomic analysis (nanoLC-MS/MS) (Lichti et al., 2014).

The convergent transcriptomic/proteomic analysis is unique because the mRNA and the protein were taken from the same animals. Therefore, in order to glean additional information from these unique datasets and aid in narrowing the many exciting targets to the most promising target, they were compared using bioinformatics. Of particular interest was whether the mRNA and protein data showed correspondence as mRNA and protein datasets often show little correspondence at the individual target level. Not only will this strategy aid in target identification, it will also aid in cross-validation of the individual core analyses.

The majority of this convergent transcriptomic/proteomic analysis chapter was published previously by Zhang and Crofton, et al., 2016[2] (co-first authors) and is used with permission.

EXPERIMENTAL PROCEDURES

Animals and behavior

Male Sprague-Dawley rats (Harlan, Houston) arrived at 21 days of age and were randomly assigned to an EC condition, 12 per cage (70 X 70 X 70 cm) with hard plastic children's toys rearranged daily, or an IC condition, singly housed in standard polycarbonate cages. The rats continued in these conditions throughout behavioral testing, which commenced at 51 days of age. The facility at UTMB is an AAALAC-accredited facility. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conform to The NIH Guide for the Care and Use of Laboratory Animals, 2011.

The overall behavioral component was a 2X2 design comparing EC vs. IC rats self-administering cocaine vs. saline. To eliminate differences in cocaine intake between EC and IC groups, rats were first food regulated down to 85% of free-feeding body

weight and trained to bar press for 45 mg banana-flavored sucrose pellets. Rats were given access to sucrose pellets for 15 min daily, with the first session being a fixed ratio 1 (FR1) schedule. The ratio was incremented daily for four additional sessions. After the FR5 session, rats were allowed to free-feed for one week prior to catheter surgery.

For surgery, rats were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. Silastic catheters were constructed in-house. The jugular vein was isolated and the catheter inserted 4.5 cm. The catheter exited through the skin on the upper back. Catheters were flushed daily with 0.1 ml of a sterile saline solution containing heparin (30.0 U/ml) and Ticarcillin (250,000 U/ml). Rats were allowed one week for recovery.

For self-administration, rats were placed in an operant chamber (Med-Associated, St. Albans, VT) and allowed to self-administer 0.5 mg/kg/infusion cocaine hydrochloride (NIDA Drug Supply Program, Research Triangle) for 2 hrs daily for a total of 14 days on an FR1 schedule of reinforcement. Rats were able to self-administer up to 30 infusions per session and were removed from the chambers at the end of the session (2 hours) or when 30 infusions were delivered. The 30-infusion limit was used to eliminate EC/IC differences in intake, which could skew the results. Tissue from rats that did not acquire self-administration was not harvested. Rats were sacrificed 3 hrs after the beginning of the self-administration session on the last day, where the nucleus accumbens tissue was dissected and flash-frozen for later processing. The left side of the nucleus accumbens of each rat was used for quantitative RNA sequencing while the right was used for quantitative proteomics analysis.

Quantitative transcriptomics study

Detailed methods can be found in (Zhang et al., 2016a). Briefly, RNA from the left NAc was purified with the RNeasy kit (Qiagen) and reverse transcribed into RNA. Adapters were ligated to “a-tailed” ends. RNA was sequenced using an Illumina HiSeq 1000 system and individual reads were mapped to each rat using Tophat (v2.0.4) and

Bowtie2 (v2.0.0.6) software packages with a reference genome (RN4). The quality of mapping was checked with FastQC (v0.9.1). The data were analyzed as FPKM and the "trimmed mean for M-values" (TMM) was used for normalization with EdgeR (v3.0.8). A likelihood ratio F-test was used to analyze the main effect of environmental enrichment and cocaine, as well as the environment X cocaine interaction.

Quantitative proteomics study

A full description of methods can be found in (Lichti et al., 2014). Briefly, the right NAc from two rats in the same group were pooled randomly for protein extraction. Tissue samples were washed in ice cold tris-buffered saline (TBS), homogenized in ice cold buffer (TBS, ph7.4, 1% Igepal-CA630 (NP-40), 1x protease inhibitor cocktail, 20mM NaF, 1 mM Na3VO4, 10 mM DTT, and 5 mM EDTA) and centrifuged (750xg, 2 min, 4°C). The pellet with the nuclear fraction was set aside while the top fraction was removed and centrifuged separately (20,000xg, 20 min, 4°C). Methanol and chloroform (v/v 1:4:1) was added to the resulting supernatant and kept at 24°C for 15-30, vortexing every 5 min, and centrifuged (16,000xg, 20 min, 4°). Acetone was added (500µl) to remove methanol and chloroform. The membrane fraction (20,000xg pellet) was treated similarly to remove lipids (i.e. membrane fraction). All fractions were finally dissolved in urea buffer (6M urea, 1% NP-40, 20mM Tris-HCl pH 7.4, 1xprotease inhibitor cocktail, 10mM DTT and 5mM EDTA) and stored at -80° until preparation for LC-MS.

For nanoLC-MS, protein extracts were digested with trypsin and incubated at 37°C overnight. The peptide mixture was extracted with a C18 Zip Tip (Pierce), eluted with 90% acetonitrile in 0.1% TFA, dried and resuspended with 5mM Tris, pH 7.4 (25µl). Peptide mixtures from each group were randomized and analyzed by nanoLC-MS/MS with a nanoLC chromatography system (Eksigent), coupled on-line to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) through a nanospray ion source. Peptide samples were injected into the trap column and eluted by gradient elution.

LC-MS/MS data were acquired with XCalibur (v2.1.0, Thermo). Total run time was 104 min. MS files were imported into Progenesis LC-MS (v4.1, Nonlinear Dynamics) for m/z and retention time alignment. The top 5 spectra for each feature were exported and database searched in PEAKS (v6, Bioinformatics Solutions) against a combined UniProtKB/SwissProt rat-mouse database (v. Sept 2013), which was appended with the common Repository of Adventitious Proteins (cRAP) contaminant database. A filtered peptide intensity list was log2 transformed in Dante-R and combined with protein abundances (RRollup). Proteins were quantified by two-way ANOVA, and p-value adjustment performed based on the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). LC-MS data files are available via ProteomeXchange with identifier PXD000990.

Secondary Bioinformatic Analyses

The Perseus software package was first used to annotate individual targets with Gene Ontology (GO) annotations, aggregate scores within each gene set, and analyze gene sets using a 2D annotation enrichment analysis (Cox and Mann, 2012). A gene set was deemed significant at $p < 0.05$. Next, an Ingenuity Pathway Analysis (IPA) Comparison Analysis was performed for lists of transcripts and proteins significantly regulated at the $p < 0.05$ cutoff level of significance for the individual primary analyses. The IPA analysis uses a Fisher's exact test to generate p-values for curated transcript/protein sets separately grouped by canonical pathways, upstream regulators, and biological functions and diseases.

Venn diagrams were used to depict significantly regulated individual mRNA/protein targets (Figure 3.2A and Figure 3.6A) in specific IPA canonical pathways and significantly regulated gene sets (Figure 3.2B-D and Figure 3.6B-D) at the $p < 0.05$ level of significance.

To analyze the data without p-value cutoffs, Gene Set Enrichment Analyses (GSEA) were used to analyze which curated gene sets showed corresponding and statistically significant changes in mRNA or protein between environmentally enriched versus isolated animals or animals that self-administered cocaine versus saline. Gene Set Enrichment Analysis results in an enrichment score: a running-sum statistic based on whether the genes in the set are enriched at the top of all the ranked genes (positive value) or at the bottom (negative value). In order to compare across gene sets, the enrichment scores are converted into normalized enrichment scores (NESs) calculated by dividing the original enrichment score by the average of enrichment scores against all permutations of the dataset.

Scatterplot data were visualized using the Tableau software package (http://www.tableau.com/).

RESULTS

The primary analyses of tissue from the NAc of enriched or isolated animals that self-administered cocaine or saline determined that environmental enrichment regulated 3393 of 14,309 transcripts ($p < 0.05$; (Zhang et al., 2016a)) and 117 of 1917 proteins (Lichti et al., 2014). Cocaine regulated 1274 transcripts and 52 proteins. This chapter is a secondary analysis of these previous proteomic and transcriptomic analyses to gain a more thorough understanding of the overall effects of environmental enrichment and cocaine as well as novel target identification.

Environmental Enrichment effects comparing mRNA and protein

The mRNA and protein data were compared using the Perseus software package for the enriched versus isolated animals. Figure 3.1A shows the comparison of mRNA and protein data for three large Gene Ontology categories: Biological Processes (GOBP), Molecular Functions (GOMF), and Cellular Compartment (GOCC). Each category

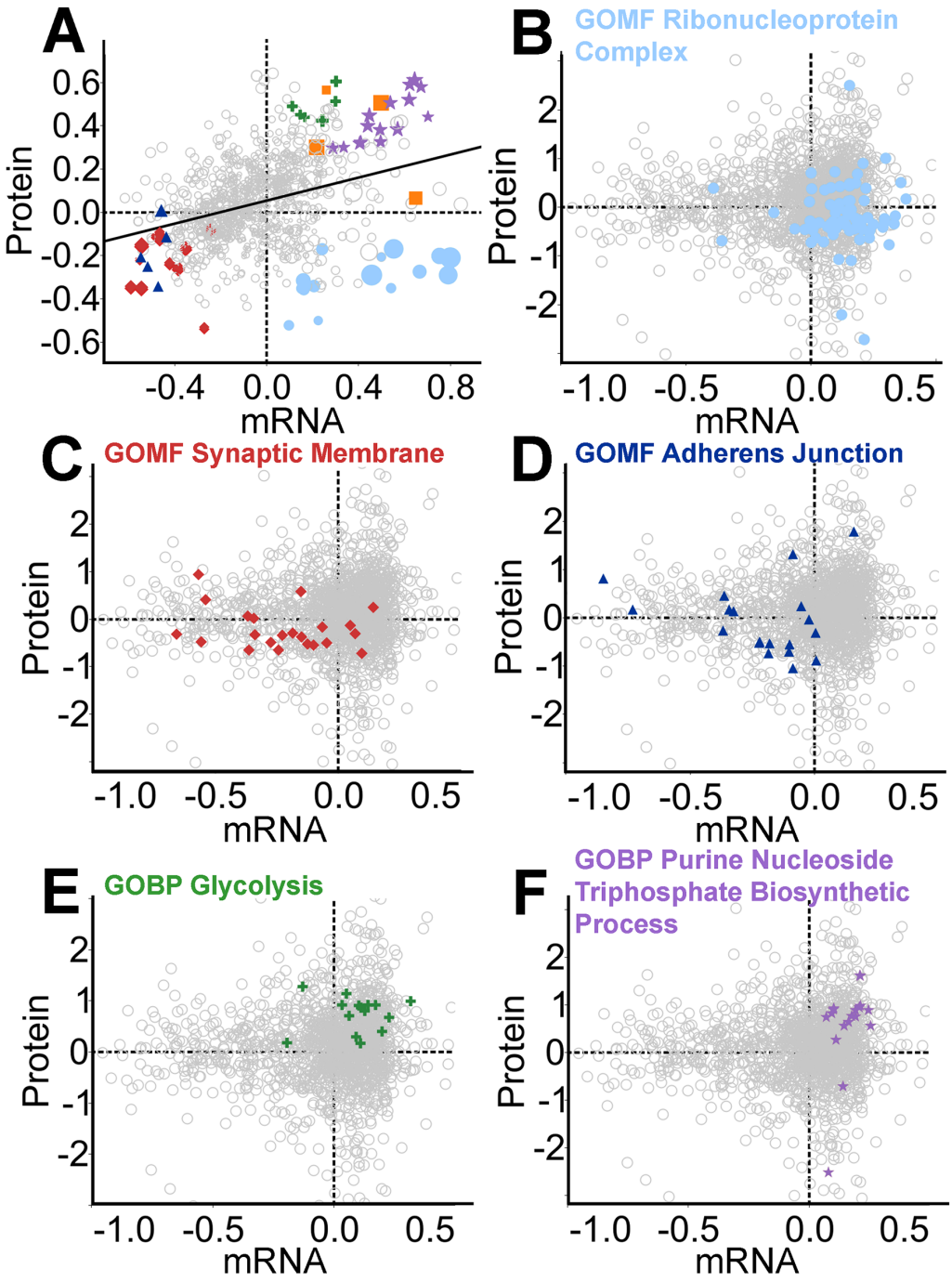

Figure 3.1 Comparison of mRNA and protein regulation by environmental enrichment at the gene set level via Perseus

(A) Correlation of all gene sets comparing mRNA and protein data. Larger symbol indicates higher –log (*p*) value for the gene set. Colors of symbols correspond to colors in panels B-F of this figure. (B) Members of the Gene Ontology Molecular Function Ribonucleoprotein Complex gene set overlaid on all genes identified with mRNA and protein analyses. (C) Members of the Gene Ontology Molecular Function Synaptic Membrane gene set highlighted. (D) Members of the Gene Ontology Molecular Function Adherens Junction gene set highlighted. (E) Members of the Gene Ontology Biological Process Glycolysis gene set highlighted. (F) Members of the Gene Ontology Biological Process Purine Nucleoside Triphosphate Biosynthetic Process gene set highlighted.

contains many gene sets or groups of genes that are segregated based on a specific biological process, molecular function, or cellular compartment. A larger circle indicates a higher –log (*p*) value for the Gene Ontology gene set. We found that for environmental enrichment, mRNA and protein Gene Ontology data show a positive correlation at the gene set level, indicated by the regression line ($R^2 = 0.109$, $p < 0.0001$, Figure 3.1A).

In addition to an overall correlation in Gene Ontology, we also found that specific gene sets with similar function or cellular compartment tended to show coordinated regulation at the mRNA and protein levels. For example, the orange circles in Figure 3.1A are clustered in the top right quadrant, indicating mRNA and protein levels were increased in the enriched animals. These data points are all related to mitochondria and include the gene sets: *mitochondrial part* (GOCC; p = 5.4E-6), *mitochondrion organization* (GOBP; p = 0.02), *mitochondrial membrane part* (GOCC; 7.5E-6), and *mitochondrial membrane part* (GOMF; 1.4E-4). Gene sets related to glycolysis (dark green) and gene sets related to the synthesis of ATP (purple) also showed increases in mRNA and increases in protein (Figure 3.1A). The individual genes from the *glycolysis* (GOBP; p = 1.9E-3) gene set are highlighted in Panel E while the genes from the *purine nucleoside triphosphate biosynthetic process* (GOBP; p = 1.6E-5) gene set are highlighted in Panel F. Not only are the similar gene sets segregated into specific quadrants related to increases in mRNA and increases in protein, but individual genes in the gene sets are also showing correspondence in mRNA and protein. These results indicate environmentally enriched animals have higher mRNA and protein related to mitochondria, ATP, and glycolysis than isolated animals.

Gene sets related to synapses and adherens junctions in Figure 3.1A show decreases in mRNA and corresponding decreases in protein in enriched animals. The gene sets *synaptic membrane* (GOMF; p = 3.2E-4) and *adherens junction* (GOMF; p = 2.4E-3) are shown in Panels C and D, respectively. These individual gene sets also show

that individual genes are decreased by environmental enrichment at both the mRNA and protein levels.

Gene sets related to ribosomes, translation, and splicing are found in the lower right quadrant, indicating enriched animals have an increase at the mRNA level but a decrease at the protein level (Figure 3.1A, light blue). The gene set *ribonucleoprotein complex* (GOMF; p = 3.0E-9) is shown in Panel B and the majority of the genes in this gene set show opposite regulation by enrichment in mRNA and protein.

In order to examine the similarities and differences in the biological functions and pathways regulated by environmental enrichment, we used Ingenuity Pathway Analysis to compare mRNA and protein data. Figure 3.2A depicts several top-ranked canonical pathways for both protein and mRNA regulated by environmental enrichment. The bar Venn diagrams illustrate the significantly regulated transcripts that were identified in the transcriptomic study only (purple), the proteins identified in the proteomics study only (blue), and the transcripts/proteins which were identified with both and showed coordinated regulation (increased in both mRNA and protein, or decreased in both mRNA and protein; light purple) and those that showed opposite regulation (increased in one analysis, decreased in the other analysis; red) for the environmental enrichment main effect. Overall, many more transcripts were quantified than proteins, leading to a greater number of regulated targets; however, there seems to be little coordinated regulation of a given target by environmental enrichment at both the mRNA and protein level (light purple), with the exception of *Mitochondrial Dysfunction*.

Despite a lack of coordinated regulation of individual targets, there was a good correspondence for significantly regulated canonical pathways, upstream regulators, and biological functions, (Panels B-D, respectively) for the environmental enrichment main effect. For example, 237 canonical pathways were significantly regulated by enrichment in the transcriptomic analysis, 106 were statistically significant in the proteomic analysis, and 82 canonical pathways were significantly regulated in both data sets (Figure 3.2B).

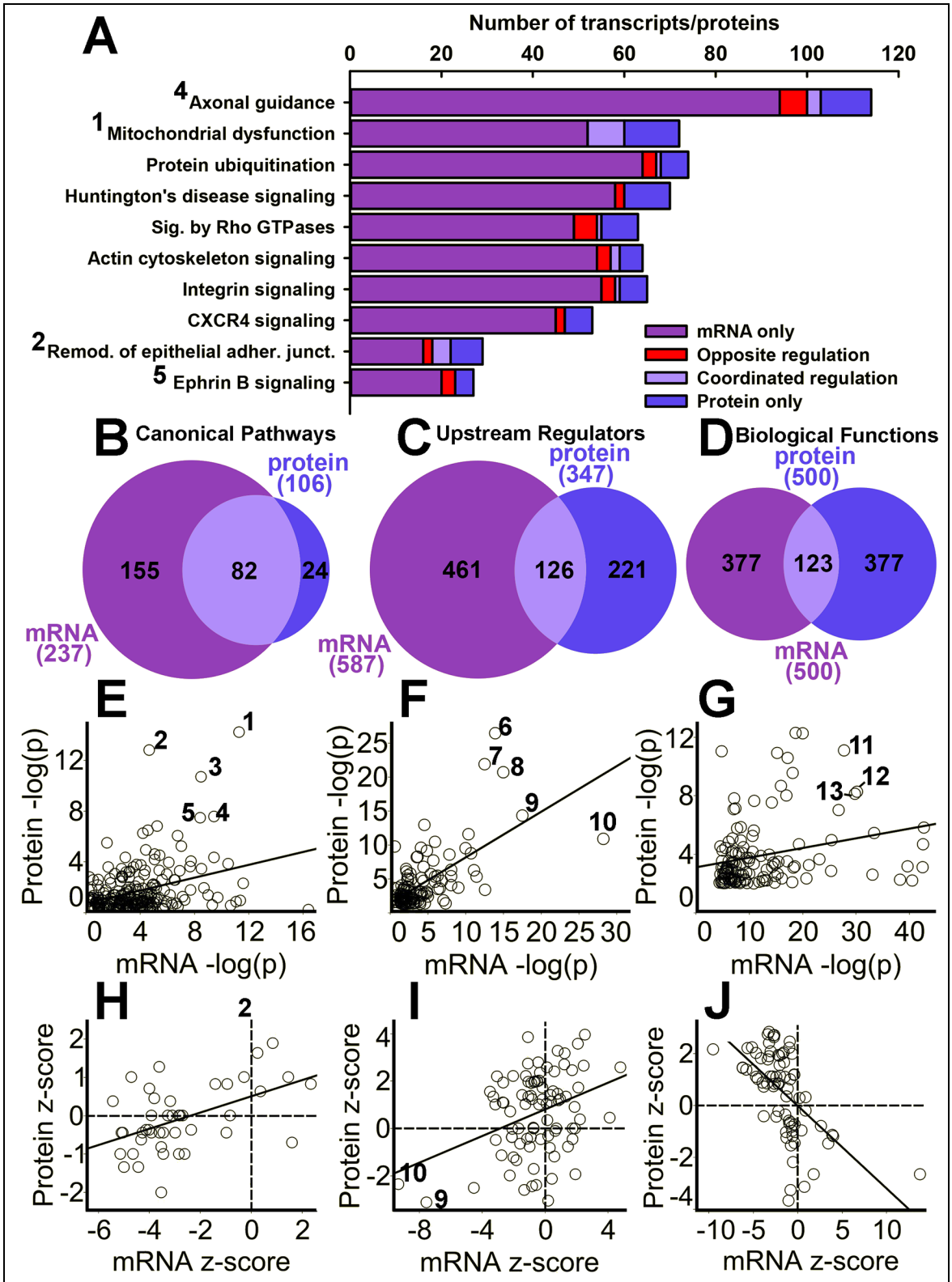

Figure 3.2 Environmental enrichment-induced protein vs. mRNA regulation at the gene set level via Ingenuity Pathway Analysis

(A) Venn diagrams showing degree of overlap of target gene regulation for some top-regulated Canonical Pathways by environmental enrichment. Dark purple or left-most part of diagram denotes number of targets regulated exclusively at the mRNA level and blue or right-most sections represent exclusive regulation at the protein level. Light purple in the middle denotes coordinated regulation of both protein and mRNA, and red in the middle represents number of oppositely regulated targets by environmental enrichment. (B-D) Venn diagrams showing the degree of overlap of the number of significant Canonical Pathways (B), Upstream Regulators (C), and Biological Functions (D) between the mRNA and protein analyses. Negative log (*p*) (E-G) and Z-score correlations (H-J) for mRNA and proteins of individual Canonical Pathways (E,H), Upstream Regulators (F,I), and Biological Functions (G,J). Numbers in A-I denote Canonical Pathways: *Mitochondrial Dysfunction* (1), *Remodeling of Epithelial Adherens Junctions* (2), *Oxidative Phosphorylation* (3), *Axonal Guidance Signaling* (4), and *Ephrin Receptor Signaling* (5); the Upstream Regulators MAPT (6), APP (7), PSEN1 (8), CD437 (9), and RICTOR (10); and the Biological Functions *Movement Disorders* (11), *Organization of Cytoplasm* (12), and *Organization of Cytoskeleton* (13).

To identify top-regulated sets for both mRNA and protein, the –log (*p*) values and the z-scores for the canonical pathways, upstream regulators, and biological functions analyses were plotted for enrichment. All three IPA analyses showed significant correlations in –log (*p*) values and z-scores for environmental enrichment (Figure 3.2E-J, $p<0.05$). Top canonical pathways from Figure 3.2E included 1) *Mitochondrial Dysfunction*, 2) *Remodeling of Epithelial Adherens Junctions*, 3) *Oxidative Phosphorylation*, 4) *Axonal Guidance Signaling*, and 5) *Ephrin Receptor Signaling*. Top upstream regulators included 6) MAPT (microtubule associated protein tau), 7) APP (amyloid precursor protein), 8) PSEN1 (presenilin 1), 9) the retinoic acid receptor gamma agonist CD437, and 10) RICTOR (rapamycin-insensitive companion of mTOR, see also Figure 3.3E-F). Top biological functions included 11) Movement Disorders, 12) Organization of Cytoplasm, and 13) Organization of Cytoskeleton.

Closer inspection of additional addiction-related canonical pathways significant in both mRNA and protein data sets for the environmental enrichment main effect underscore the lack of correspondence at the individual target level. For example, the *CREB Signaling Pathway* (mRNA –log (*p*) = 6.93; protein –log (*p*) = 3.32; Figure 3.3A-B), and the *Gαq Signaling Pathway* (mRNA –log (*p*) = 2.42; protein –log (*p*) = 1.69; Figure 3.3C-D) were significantly regulated by environmental enrichment, but none of the individual targets were coordinately regulated in protein and mRNA (for AC, PLC

and Gα, different members of these groups were regulated at the protein and mRNA levels). This is illustrated in Figure 3.3 where the colored symbols (green for downregulated and red for upregulated) are not the same between the mRNA (Panels A and c) and the protein (Panels B and D) for environmental enrichment. If the symbol is white, it was not significantly regulated by environmental enrichment. These data are

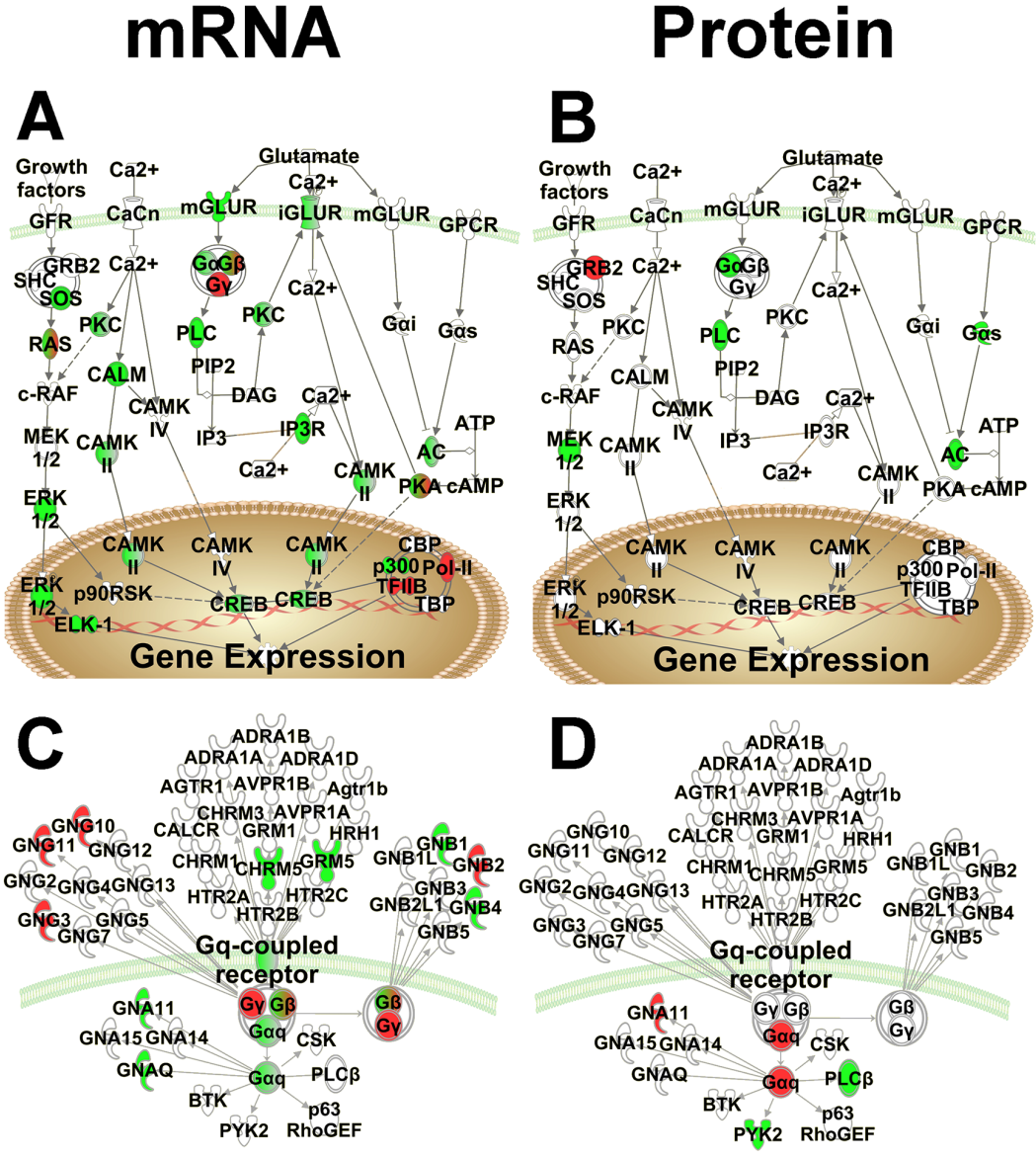

Figure 3.3 Environmental enrichment regulation of specific Canonical Pathways at the mRNA level (left) and protein level (right)

(A-B) Comparison of regulated targets for the CREB signaling pathway. Red denotes upregulation by environmental enrichment and green denotes down-regulation. (C-D) Comparison of regulated targets for G-protein coupled receptor signaling.

further showing that the significantly regulated individual mRNAs and proteins do not overlap for environmental enrichment but some of the same pathways are identified in both analyses.

We analyzed the normalized enrichment scores (NES) of conserved Transcription Factor Targets (TFT) gene sets from the Molecular Signature Database (MSigDB) (Subramanian et al., 2005) regulated by environmental enrichment. We were particularly interested in coordinated regulation of transcription factor targets because several transcription factors have been previously identified as involved in the protective addiction phenotype of environmental enrichment (Green et al., 2010; Pavlovsky et al., 2013; Zhang et al., 2014). When we compared the NESs for the mRNA vs. protein (Figure 3.4A), we identified several coordinately regulated transcription factor target sets for the environmental enrichment main effect (shown in Panels B-F). Specifically, the transcription factors ATF6 (mRNA NES = −1.52, p = 0.02; protein NES = −1.57, p = 0.03), OCT1 (mRNA NES = −1.49, p = 0.006; protein NES = −1.5, p = 0.03), VDR (mRNA NES = −1.48, p = 0.004; protein NES = −1.48, p = 0.04), MEF2 (mRNA NES = −1.52, p = 0.008; protein NES = −1.47, p = 0.04), and YY1 (mRNA NES = −1.42, p = 0.08; protein NES = −1.63, p = 0.01) showed coordinated regulation of protein and mRNA by environmental enrichment.

Cocaine effects comparing mRNA and protein

Using the Perseus software package we compared the mRNA and protein Gene Ontology data for the animals that self-administered cocaine vs. saline. The correspondence between the mRNA and protein data for three large Gene Ontology categories (Biological Processes (GOBP), Molecular Functions (GOMF), and Cellular Compartment (GOCC)) is shown in Figure 3.5A for the cocaine main effect. We found a positive correlation between the mRNA and protein data for the main effect of cocaine

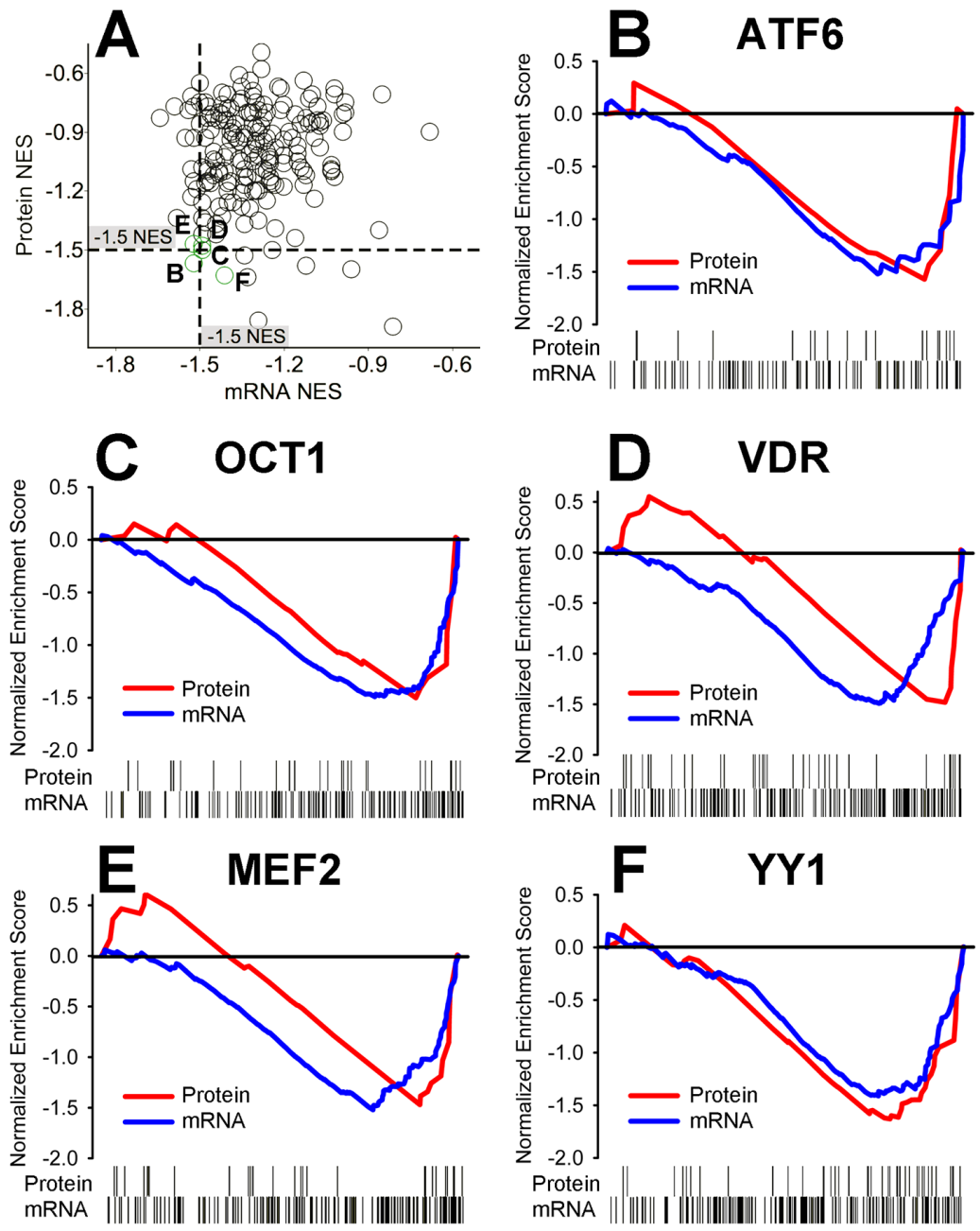

Figure 3.4 Environmental enrichment-induced regulation of target genes of specific transcription factors

(A) Gene Set Enrichment Analysis correlation of normalized enrichment scores (NES) for top-regulated transcription factor target genes. The labels B-F on Panel A label the green circles that correspond to the transcription factor target genes shown in Panels B-F of this figure. (B-F) Running sum NESs for ranked targets that are conserved targets of ATF6, OCT1, VDR, MEF2, and YY1, respectively. Downward deflection of the graph indicates the targets of these transcription factors are downregulated in enriched rats in the protein (red) or mRNA (blue) data. Vertical ticks along the bottom of each panel indicate where the gene targets appear in the ranked list of genes for the protein and the mRNA data.

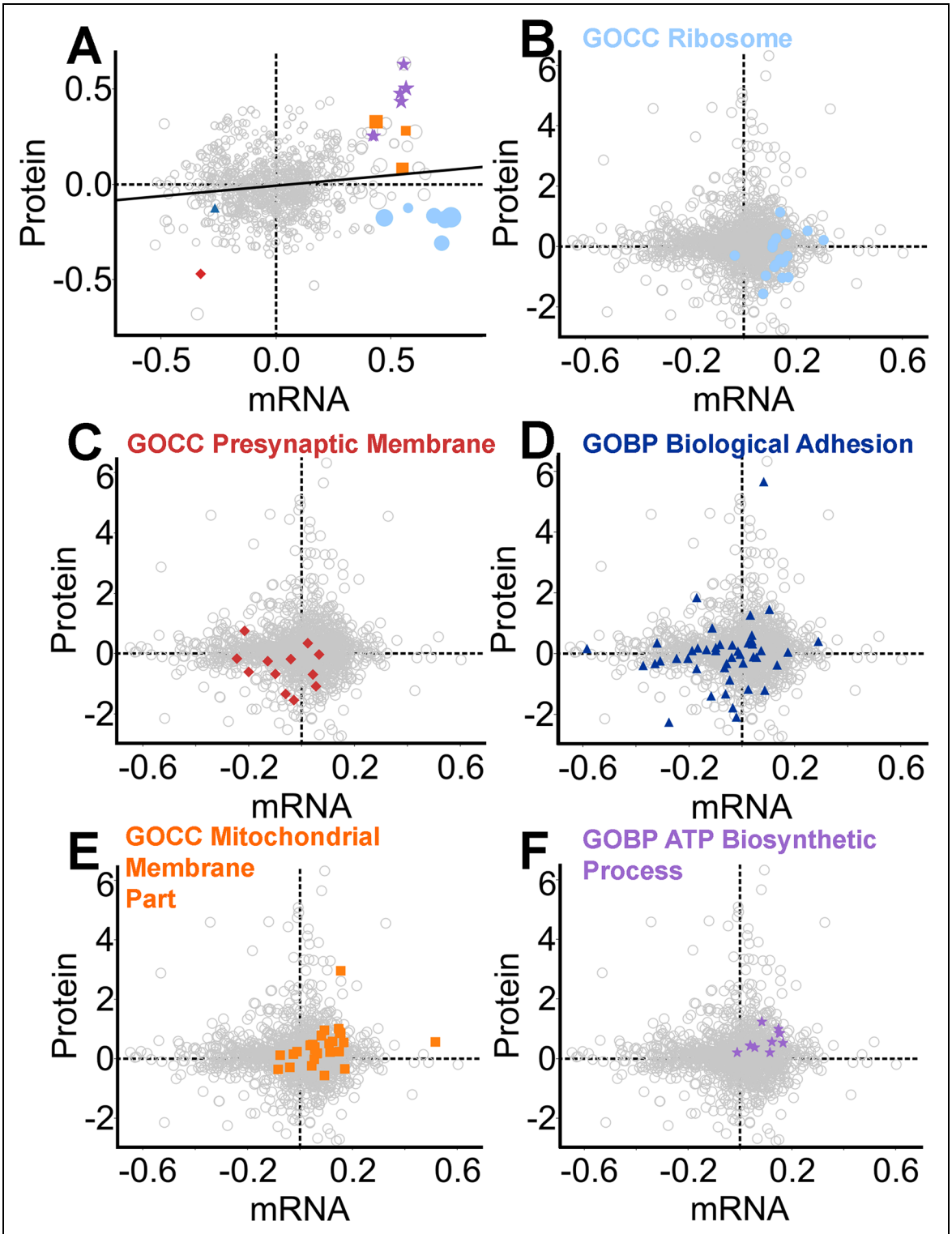

Figure 3.5 Comparison of mRNA and protein regulation by cocaine at the gene set level via Perseus

(A) Correlation of all gene sets comparing mRNA and protein. Larger symbol indicates higher –log (p) value for the gene set. Colors of symbols correspond to colors in panels B-F of this figure. (B) Members of the Gene Ontology Cellular Compartment Ribosome gene set highlighted overlaid on all genes identified with mRNA and protein analyses. (C) Members of the Gene Ontology Cellular Compartment Presynaptic Membrane gene set highlighted. (D) Members of the Gene Ontology Biological Process Biological Adhesion gene set highlighted.

(R^2 = 0.02; p < 0.0001); however, the correlation for cocaine is less than that of environmental enrichment (R^2 = 0.109, p < 0.0001, Figure 3.1A). This likely reflects the greater number of targets regulated by enrichment (mRNA = 3393; protein = 117) vs. cocaine (mRNA = 1274; protein = 52).

As with environmental enrichment, we found clusters of the same categories of gene sets including gene sets involved in ribosomes, synapses, adhesion, mitochondrial, and ATP synthesis for the cocaine main effect. Ribosomal gene sets for cocaine are found to have increased mRNA levels but decreased protein levels similar to environmental enrichment. The GOCC gene set for *Ribosome* is shown in Figure 3.5B (p = 6.2E–6). Cocaine increased mRNA and protein targets in mitochondria and ATP synthesis gene sets *Mitochondrial membrane part* (GOCC; p = 5.5E–6) and *GOBP ATP biosynthetic process* (p = 6.3E–4; Figure 3.5E–F, respectively). Gene sets related to synapses (e.g. *Presynaptic membrane*, p = 8.2E–3; Figure 3.5C) and adhesion (e.g. *Biological adhesion*, p = 0.019; Figure 3.5D) show coordinated decreases in mRNA and protein for the cocaine main effect.

We explored the similarities and differences in the biological functions and pathways regulated by cocaine in the mRNA and protein data using Ingenuity Pathway Analysis. Compared to the environmental enrichment data, there was much less opposite regulation between mRNA and protein in top-ranked canonical pathways (Figure 3.6A). We found as with enrichment that the individual mRNAs and proteins within a specific pathway showed little correspondence, but there was more overlap at the canonical pathway level (34 of 70 significant protein pathways; Figure 3.6B). There was considerably less overlap for upstream regulators and biological functions (Figure 3.6C-

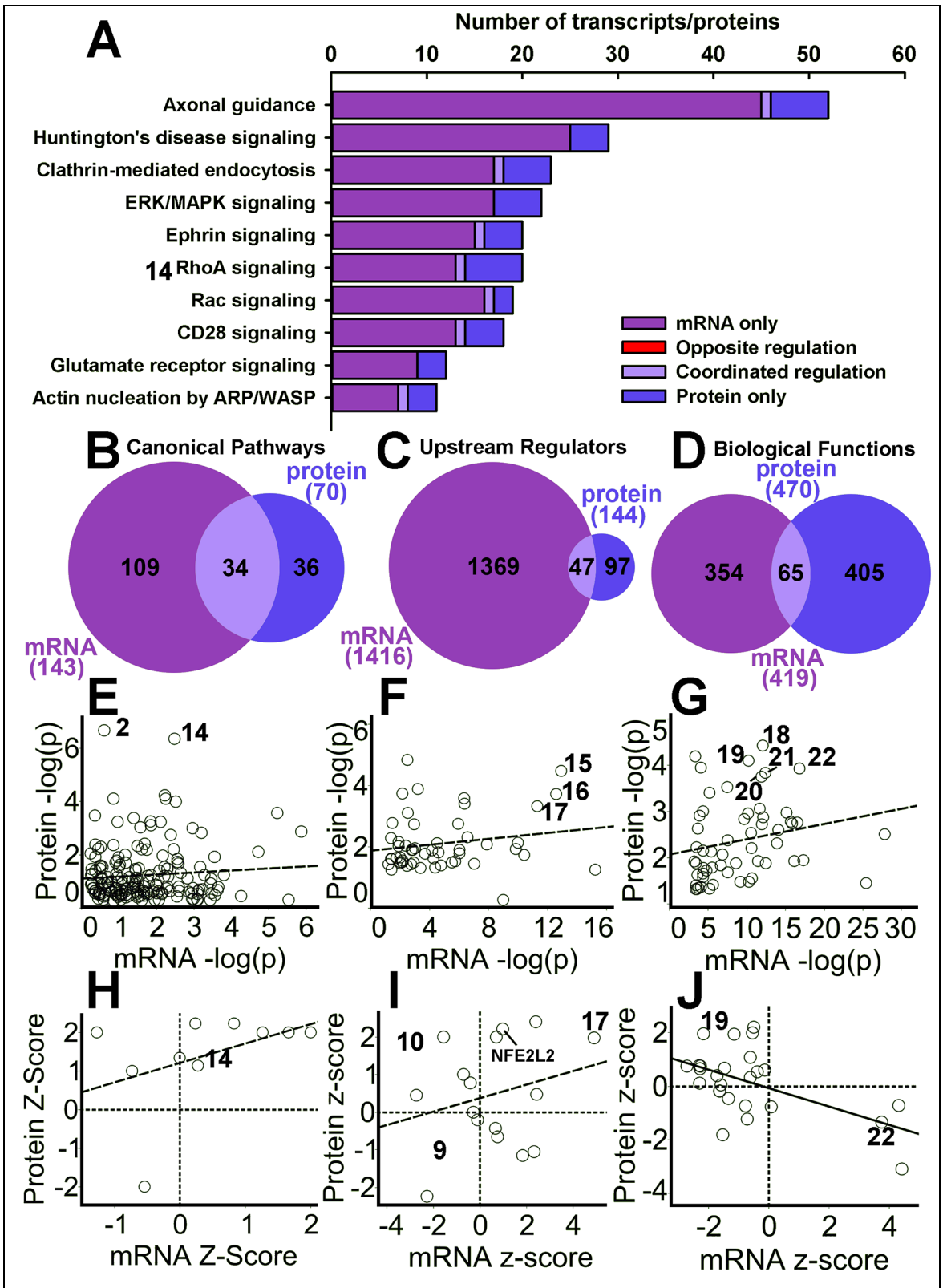

Figure 3.6 Cocaine-induced protein vs. mRNA regulation at the gene set level via Ingenuity Pathway Analysis

(A) Venn diagrams showing degree of overlap of target gene regulation for top-regulated Canonical Pathways by the cocaine main effect. Dark purple or left-most part of diagram denotes number of targets regulated exclusively at the mRNA level and blue or right-most sections represent exclusive regulation at the protein level. Light purple denotes coordinated regulation of both protein and mRNA, and red represents number of oppositely regulated targets. B-D) Venn diagrams showing the degree of overlap of the number of significant Canonical Pathways (B), Upstream Regulators (C), and Biological Functions (D) between the mRNA and protein analyses for the cocaine main effect. Negative log (*p*) scores (E-G) and Z-score correlations (H-J) for mRNA and proteins of individual Canonical Pathways (E,H), Upstream Regulators (F,I), and Biological Functions (G,J). Numbers in A-J denote Canonical Pathways: Remodeling of Epithelial Adherens Junctions (2), and RhoA Signaling (14); the Upstream Regulators CD437 (9), RICTOR (10), NFE2L2, HTT (15), APP (16), and Forskolin (17); and the Biological Functions Disorder of Basal Ganglia (18), Size of Body (19), Huntington's Disease (20), Neuromuscular Disease (21), and Movement Disorders (22).

D). The canonical pathway *RhoA signaling* was a top-scoring canonical pathway (Figure 3.6E, number 14). Top upstream regulators for cocaine included 15) huntingtin (HTT), 16) amyloid precursor protein (APP) and 17) the cAMP activator forskolin (see also Figure 3.7E-F). Top biological functions for cocaine included 18) *Disorders of the basal ganglia*, 19) *Size of body*, 20) *Huntington's disease*, 21) *Neuromuscular disease*, and 22) *Movement disorders*. Analyzing the –log (*p*) values for protein vs. mRNA for Canonical Pathways, Upstream Regulators, and Diseases and Biological Functions did not produce significant correlations for –log (*p*) values from cocaine regulation (Figure 3.6E-G), and only the Diseases and Biological Functions analysis produced a significant z-score correlation (Figure 3.6J).

We examined pathways from IPA that were significantly regulated by cocaine at the mRNA and the protein level, and we found that the significant canonical pathway for *Regulation of actin-based motility by Rho* was significant for protein (–log (*p*) = 3.38, Z-score = 2.0) and mRNA (–log (*p*) = 1.41, Z-score = 1.7) yet had only ARPC5L in common (Figure 3.7A-B). The *ERK/MAPK signaling* pathway had no overlap at the individual target level for the cocaine main effect (protein –log (*p*) = 3.12, mRNA –log (*p*) = 1.87; Figure 3.7C-D). These data suggest that the mRNA and protein analyses are

not identifying the same individual genes as contributing to the cocaine or the environmental enrichment effects but both levels of analysis are pointing toward similar pathways and gene sets.

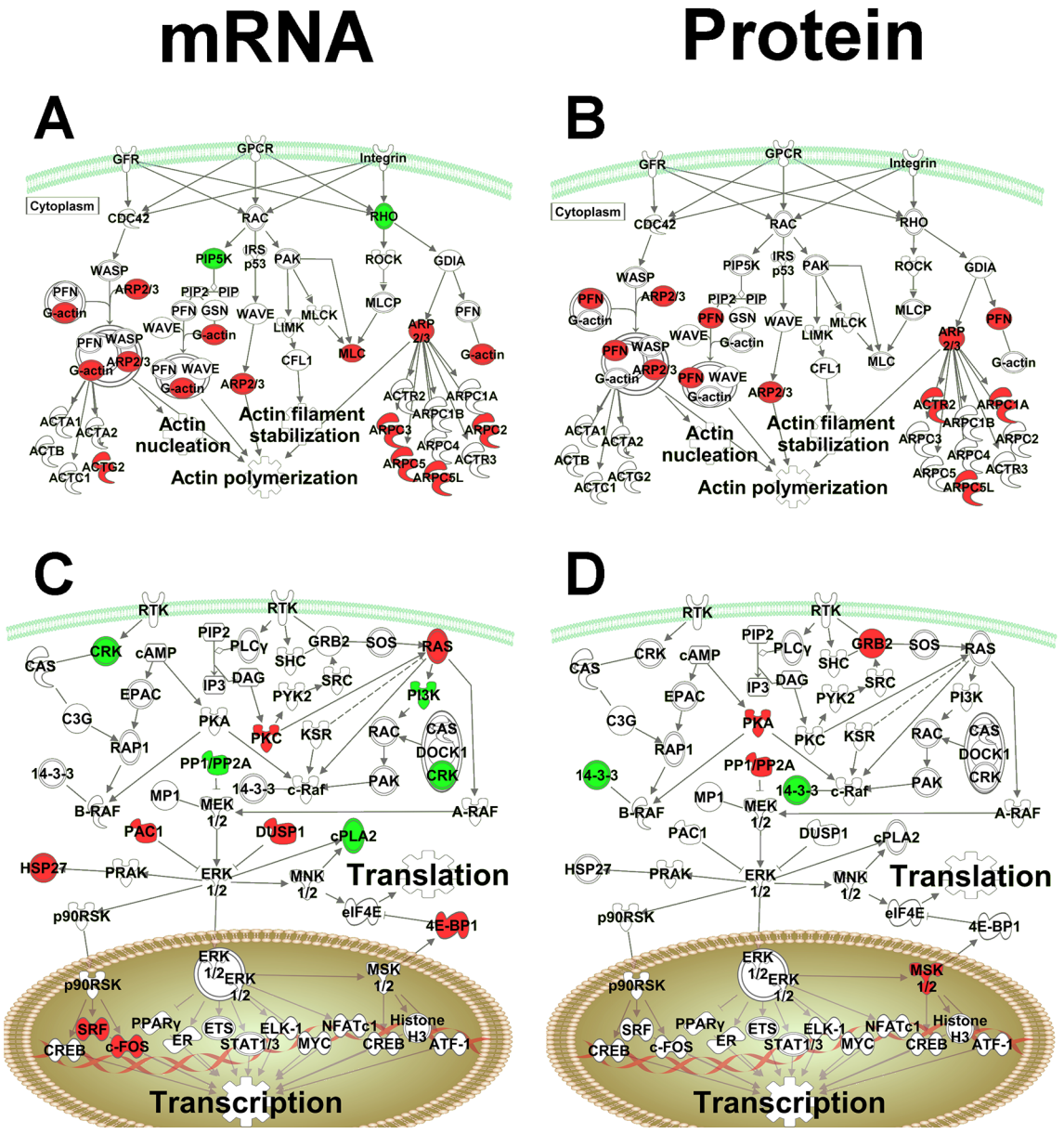

Figure 3.7 Cocaine regulation of specific Canonical Pathways at the mRNA level (left) and protein level (right)

(A-B) Comparison of cocaine regulated targets for the Actin pathway. Red denotes upregulation by cocaine and green denotes downregulation. (C-D) Comparison of regulated targets for *ERK/MAPK signaling* cocaine.

IDENTIFICATION OF TARGET PATHWAY: PI3K/AKT SIGNALING

The goal of this convergent transcriptomic/proteomic approach is to determine novel targets for neuropsychiatric disease through investigation of environmental enrichment and cocaine. One such pathway identified is *PI3K/AKT signaling*. The canonical pathway *PI3K/AKT signaling* was significantly regulated by environmental enrichment at the mRNA and protein levels with IPA (EC mRNA –log (*p*) = 3.21; EC protein –log (*p*) = 2.02). *PI3K/AKT signaling* was also significantly regulated by cocaine at the protein level (cocaine protein –log (*p*) = 1.91) and the core PI3K/AKT pathway was regulated by cocaine at the mRNA level (cocaine mRNA –log (*p*) = 1.69). *PI3K/AKT signaling* is shown in Figure 3.8 overlaid with transcripts significantly regulated by

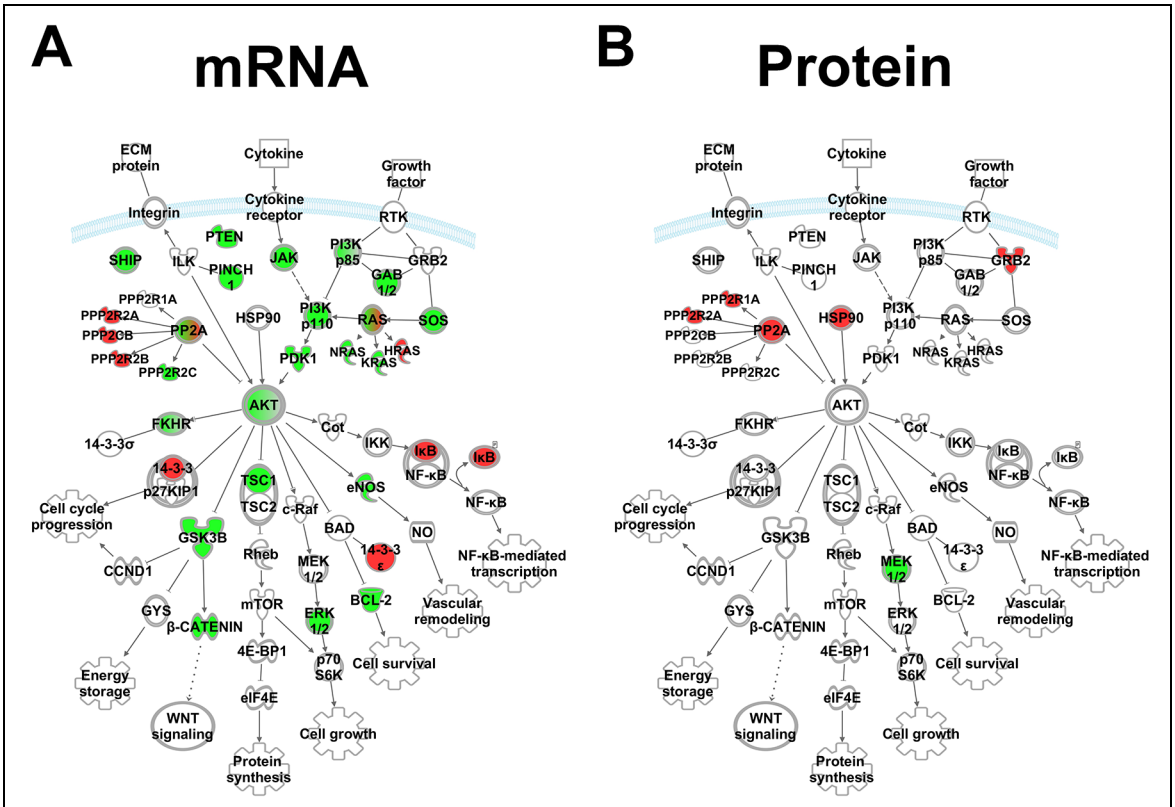

Figure 3.8 Environmental enrichment regulation of the PI3K/AKT signaling canonical pathway at the mRNA (left) and protein level (right)

Red denotes upregulation by environmental enrichment and green denotes down-regulation.

environmental enrichment at the mRNA or protein levels. Downstream targets of AKT were also regulated by environmental enrichment and cocaine at the mRNA level, as AKT was identified as an upstream regulator (EC mRNA –log (p) = 2.123; cocaine mRNA –log (p) = 2.897). In addition to IPA, the PI3K/AKT pathway was also identified by GSEA. GSEA identified the curated gene set *Reactome PI3K AKT Activation* as significantly downregulated regulated by the environmental enrichment main effect (mRNA NES = –1.6, p = 0.03, Figure 3.9).

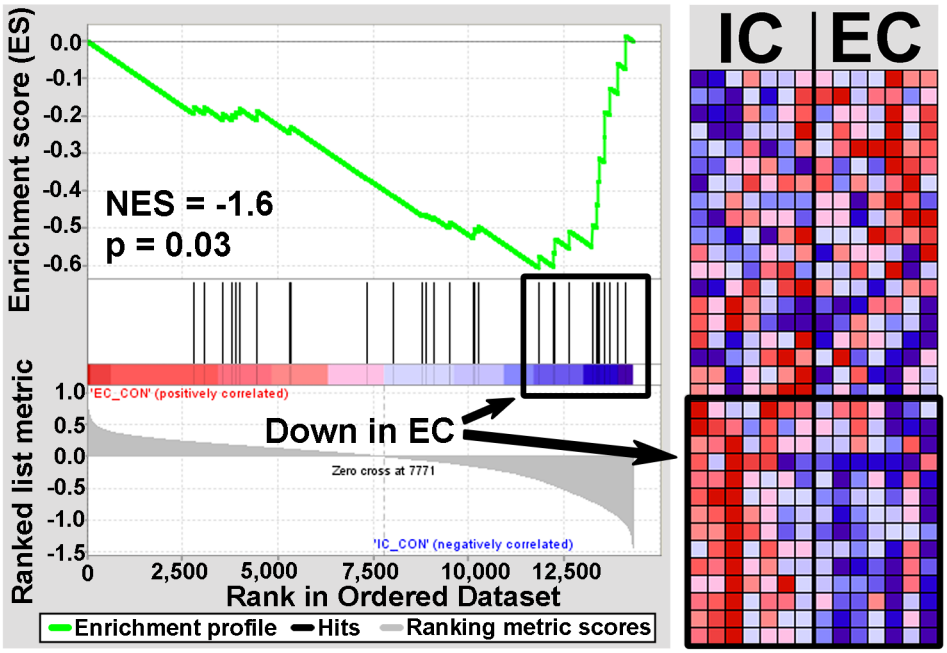

Figure 3.9 Transcriptomic analysis via GSEA identifies PI3K AKT Reactome pathway as downregulated in EC rats

The further investigation of this pathway reveals that a downstream target of AKT, glycogen synthase kinase 3 beta (GSK3β), is differentially regulated by environmental enrichment and cocaine (Figure 3.9). At the mRNA level, GSK3β is significantly regulated by environmental enrichment ($p < 0.001$) and significantly regulated by the interaction between enrichment and cocaine (p = 0.037). The cocaine main effect is not significant for GSK3β mRNA (p = 0.25).

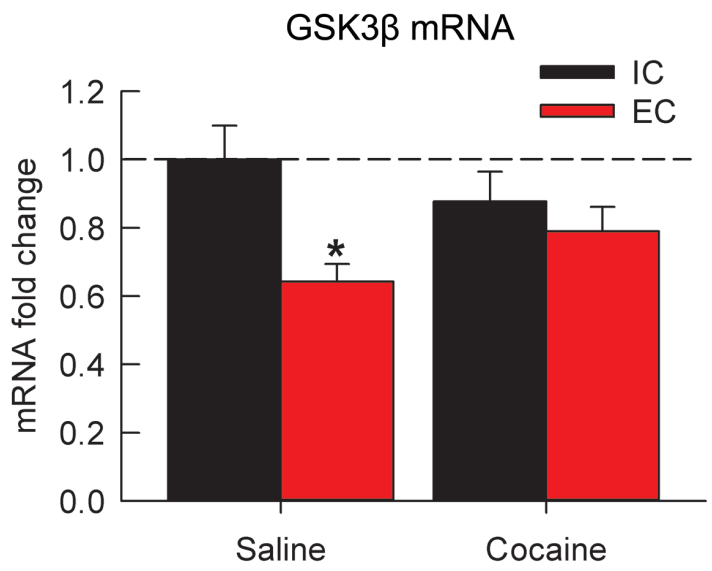

Figure 3.10 mRNA of GSK3β is differentially regulated by environmental enrichment and cocaine

mRNA fold change normalized to IC saline rats. Red bars indicate enriched rats. Black bars indicate isolated rats. mRNA fold change data from Zhang et al., 2016b.

The convergent transcriptomic/proteomic analysis identifies AKT signaling as a possible target for further investigation, especially the AKT downstream target, GSK3β. Thus, Strategy 1 leads to the development of the hypothesis that GSK3β in the nucleus accumbens is involved in the regulation of behavior.

DISCUSSION

These results show clearly that complementary proteomic and transcriptomic studies can identify similar gene sets, canonical pathways, and upstream regulators even when there is little correspondence of regulation among individual transcripts vs. proteins. This underscores the robustness of the bioinformatic approaches in the face of the high Type I and Type II error inherent to large-scale transcriptomics and proteomics. However, these results also demonstrate that manipulations producing more robust regulation (in this case, environmental enrichment regulated 3393 transcripts and 117 proteins) result in greater correspondence among pathways than less robust manipulations

(cocaine only regulated 52 proteins and 1274 transcripts), as one might expect. Overall, more transcripts were identified than proteins, which is most likely due to factors idiosyncratic to both the next generation RNA sequencing and the nanoLC-MS/MS. However, even though 1) more mRNA transcripts were included in the pathway analysis than protein, and 2) genes are differentially regulated in mRNA and protein levels, the comparison analysis shows that the results concur at the gene set level despite these differences.

The advantage of assessing both the proteome and transcriptome from the same set of animals is that the gene sets significantly regulated at both levels are most likely to be substantive. This is most evident when looking at cocaine effects, as so much is already known about this drug. Several of the top pathways regulated at both the mRNA and protein levels have been identified previously as being important for cocaine neuroplasticity, including ERK/MAPK signaling (Berhow et al., 1996; Pan et al., 2011), RhoA signaling (Kim et al., 2009), RAC signaling (Dietz et al., 2012), Ephrin signaling (Yue et al., 1999), and glutamate signaling (Garcia-Keller et al., 2016). Additionally, the upstream regulator forskolin was identified by both mRNA and protein analyses. Forskolin is an activator of the transcription factor CREB, which has been shown to be an important mediator of cocaine self-administration (Green et al., 2010). Because the cocaine data identify so many previously validated mechanisms, we can have the utmost confidence in the environmental enrichment results.

Because environmental enrichment produces a protective addiction phenotype (Green et al., 2002; Thiel et al., 2009; Green et al., 2010; Chauvet et al., 2012), identifying molecular mechanisms contributing to this phenotype will likely provide new mechanisms for the prevention of and resilience to addiction behavior. The current data suggest several additional avenues for possible future therapeutic development, including PI3K/AKT signaling. Several of the pathways regulated at the protein and mRNA level for environmental enrichment fall into the category of cell to cell cytoskeletal

organization, including *Axonal guidance*, *Actin cytoskeleton signaling*, *Remodeling of epithelial adherens junctions*, and *Ephrin B signaling*. Several significant gene sub-sets within axonal guidance underscore its importance, including *Signaling by Rho GTPases*, *CXCR4 signaling*, and *Ephrin B signaling*. Energy metabolism was a recurring theme, with *Mitochondrial dysfunction* and *Oxidative phosphorylation* pathways being regulated. Other pathways are involved with intracellular signaling cascades, particularly *Gα12/13 signaling*, *Gβ/γ signaling*, *RhoA signaling*, and *CREB signaling*. These pathways have many "druggable" targets for pharmacotherapeutic development.

This discovery-based convergent strategy (Strategy 1) identified *PI3K/AKT signaling* as a promising pathway for further analysis. One downstream target of AKT, GSK3β, was differentially regulated by environmental enrichment and cocaine, suggesting a role in the nucleus accumbens in the protective depression and addiction phenotypes of environmental enrichment. This target needs to be manipulated *in vivo* to investigate this role (Chapter 4 validates the target GSK3β).

Overall, it is interesting that environmental enrichment and cocaine have similar effects at the gene set level. For example, enrichment (Figure 3.1A) and cocaine (Figure 3.5A) both increase mRNA and protein for energy metabolism constituents. Both manipulations decrease mRNA and protein for adherens junctions and synaptic constituents. Additionally, both manipulations increase mRNA expression but at the same time decrease protein expression of constituents of ribonucleoprotein complexes. Using heat maps of expression to draw conclusions, we interpret these results as a reflection of the IC saline group being qualitatively different from the other three groups. Thus, environmental enrichment and cocaine self-administration both tax the idle brain in similar ways.

This idea supports our novel hypothesis that environmental enrichment is a very mild form of stress that inoculates the brain against addiction (Chapter 2) (Crofton et al., 2015). Inoculation stress is the process of developing resilience to future stressful events

by first being exposed to mildly stressful experiences early in life. Environmental enrichment constitutes a chronic mild stress situation as the animals live in a complex environment, which is changed daily, and they interact non-aggressively with conspecifics. This experience early in life (P21-P51) inoculates enriched rats against subsequent stressors and/or drugs of abuse, creating a protective addiction phenotype that is a non-drug, non-surgical, and non-genetic manipulation (Crofton et al., 2015).

For this experiment, enriched cocaine rats were housed with enriched saline rats, so we cannot preclude the possibility of a "rub-off" effect between cocaine and saline rats. However, the return of the rats to the homecage after self-administration was coincident with the daily toy change, when saline and cocaine enriched rats all exhibit extremely high locomotor activity.

In conclusion, this chapter demonstrates a convergence in results from parallel transcriptomics and proteomics, and additionally demonstrates that the combined analysis of these data can help to narrow the many exciting leads from robust manipulations such as environmental enrichment and cocaine self-administration to one signaling pathway, AKT signaling, and one most promising target, GSK3β.

4. TARGET VALIDATION OF STRATEGY 1: GLYCOGEN SYNTHASE KINASE 3 BETA MIMICS SUSCEPTIBLE PHENOTYPE AND ALTERS NEURONAL ACTIVITY OF TONICALLY ACTIVE NEURONS[3]

The previous chapter utilized Strategy 1 in this dissertation (i.e. a discovery-based convergent transcriptomic/proteomic bioinformatic strategy), to identify a novel target for comorbid cocaine use disorder and mood disorders. *PI3KAKT signaling* was significantly regulated by environmental enrichment and cocaine at the mRNA and protein levels. AKT was identified as an upstream regulator of enrichment and cocaine at the mRNA level. A downstream target of AKT, GSK3β, was differentially regulated by environmental enrichment and cocaine at the mRNA level. These results indicate a role for GSK3β in the underlying mechanism of the protective enrichment phenotypes. However, this is a discovery-based approach and is not causal evidence for a role of GSK3β. Therefore, this chapter manipulates GSK3β *in vivo* in the nucleus accumbens shell to investigate its causal role underlying anxiety-like, depression-like, and addiction-related behaviors. This chapter was peer-reviewed and published in the journal *Neuropharmacology* by Crofton et al. 2017; it is reproduced here with permission[3].

Intracellular kinase signaling cascades, activated through a variety of mechanisms, have proven important mediators of NAcSh function, and by extension, the etiology of neuropsychiatric disorders. Specifically, the ERK/MAPK, PKA, and PKC signaling cascades have been studied in the NAcSh with success (Self et al., 1998;

[3] Reprinted from Neuropharmacology, 117, Elizabeth J. Crofton, Miroslav N. Nenov, Yafang Zhang, Federico Scala, Sean A. Page, David L. McCue, Dingge Li, Jonathan D. Hommel, Fernanda Laezza, Thomas A. Green, Glycogen synthase kinase 3 beta alters anxiety-, depression-, and addiction-related behaviors and neuronal activity in the nucleus accumbens shell, 49-60, 2017, with permission from Elsevier. doi: 10.1016/j.neuropharm.2017.01.020.

Schroeder et al., 2008; Ortinski et al., 2015). The AKT/GSK3β pathway has also garnered particular attention for its role in dopamine signaling, the actions of antipsychotic drugs, and even responses to addictive drugs, especially in the nucleus accumbens (Perrine et al., 2008; Beaulieu et al., 2009; Nwaneshiudu and Unterwald, 2010; Beaulieu et al., 2011; Wilkinson et al., 2011; Miller et al., 2014).

GSK3β was originally discovered for its role in glycogen synthesis but has since been implicated in a variety of cellular processes (Wildburger and Laezza, 2012), and dysregulation of this kinase has been implicated in bipolar disorder and neurodegenerative disorders (Grimes and Jope, 2001; Jope, 2011). One of the mechanisms of action of lithium, the commonly prescribed mood stabilizer, is inhibition of GSK3β (Klein and Melton, 1996; Stambolic et al., 1996). Heterozygous GSK3β knockout mice show reductions in depression-like behavior similar to the effects of lithium (O'Brien et al., 2004). Drugs of abuse, especially cocaine, can modulate levels of GSK3β in the NAc (Perrine et al., 2008) and GSK3β is involved in cocaine-induced hyperactivity, cocaine sensitization, cocaine reward memory, and cocaine conditioned place preference (Miller et al., 2009; Miller et al., 2014; Shi et al., 2014). Previous studies indicate that the role of GSK3β is highly dependent on brain region and even cell type as global knockdown may not have the same effects as regional or even cell-type specific knockdown (Latapy et al., 2012; Urs et al., 2012; Zhou et al., 2012). Thus, GSK3β has therapeutic potential for comorbid depression and addiction, but knowledge gaps exist on its brain region specific mechanism of action.

The role of GSK3β in cocaine self-administration, the addiction paradigm with the most face validity, is so far lacking. Additionally, few studies have examined anxiety and depression behaviors along with addiction-related behaviors in the same animals. The current study therefore explores anxiety-like, depression-like, and addiction-related behaviors in the same animals following knockdown of GSK3β in the NAcSh of rats.

The environmental enrichment manipulation combines novelty, exercise, and social contact to produce robust protective depression and addiction phenotypes (Green et al., 2002; Green et al., 2010). Enrichment increases the ratio of phosphorylated (inactive) to total GSK3β in the hippocampus and cortex (Hu et al., 2013) and environmental enrichment is able to reverse cognitive deficits caused by constitutively active expression of GSK3 in mice (Pardo et al., 2015). In the discovery-based convergent transcriptomic/proteomic analysis of the previous chapter, AKT signaling was regulated at both the mRNA and protein level by environmental enrichment. Additionally, the downstream target of AKT, GSK3β, showed differential regulation by enrichment and cocaine. Therefore, we hypothesized that GSK3β may be involved in the regulation of behavior in the nucleus accumbens.

In order to analyze the role of GSK3β specifically in the NAcSh in behavior relevant to affective disorders and drug addiction, we designed and constructed a novel adeno-associated viral vector (AAV2), which uses RNA interference to knockdown GSK3β in adult rats and allows for prolonged knockdown of GSK3β in the adult brain. We aimed to manipulate GSK3β separate from environmental enrichment and isolation to determine if it is a protective or a susceptibility factor, therefore we injected the GSK3β shRNA vector into pair housed rats and conducted anxiety-like and depression-like behavioral tests as well as cocaine self-administration. Pair housed rats are an intermediate phenotype between enrichment and isolation as discussed in Chapter 2 (Crofton et al., 2015).

The nucleus accumbens is heavily implicated in the control of emotional behavior and reward as discussed in Chapter 1 (Pontieri et al., 1995; Pliakas et al., 2001; Green et al., 2006; Nestler and Carlezon, 2006; Larson et al., 2011). As part of the ventral striatum, the nucleus accumbens has as its sole output two major populations of medium spiny neurons (MSNs) whose activity is modulated by a population of mostly cholinergic tonically active interneurons (TANs) (Lenz and Lobo, 2013). Despite comprising 1-5%

of the total population of neurons in the NAc, TANs play important roles in reward prediction, task attention, memory, addiction, and aversive behaviors (Aosaki et al., 1994; Apicella, 2002; Anagnostaras et al., 2003; Furey et al., 2008; Williams and Adinoff, 2008; Lenz and Lobo, 2013). TANs control MSN activity and are particularly responsive to salient reward-related stimuli (Morris et al., 2004). Early studies have provided evidence for a role of TANs in cocaine addiction with immunotoxin mediated cell ablation resulting in increased sensitivity to cocaine (Hikida et al., 2001) and preventing behavioral abnormalities associated with cocaine induced by centrally active acetylcholinesterase inhibitors in the NAc (Hikida et al., 2003). More recently, studies using optogenetics confirmed that selective inhibition of TANs results in suppression of cocaine induced behaviors (Witten et al., 2010), further confirming the pivotal role of these cells in reward behavior and addiction. At the circuit level, activation of TANs has been shown to elicit both fast glutamatergic transmission (Higley et al., 2011) and GABAergic inhibition of MSNs, the latter coincident with synchronous cholinergic activation and sufficient to pause MSNs firing (English et al., 2012). These studies suggest a complex role of these cells in the NAc circuit that deserves further investigation.

To provide correlative functional outcomes to the behavioral studies, we investigated the role of GSK3β on spontaneous firing and intrinsic excitability of tonically active neurons (TANs), comparing electrophysiological properties of these neurons between GSK3β knockdown animals versus control animals.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, Houston, TX) were obtained at 21-days-old (electrophysiology) or 225-250 g (behavior) and maintained in a controlled environment (temperature, 22°C; relative humidity; 50%; 12h light/dark cycle, lights on 0600h) in an

Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved colony in standard polycarbonate cages with ad libitum access to food and water except during surgery and behavioral experiments. All surgical procedures and experiments conformed to the NIH Guide for the Care and Use of Laboratory Animals and approved by The University of Texas Medical Branch Institutional Animal Care and Use Committee.

TIMELINE OF BEHAVIOR FOLLOWING GSK3β KNOCKDOWN

Rats in the behavioral cohort underwent stereotaxic vector injection one week after arrival. Three weeks later, rats underwent a battery of behavioral tests beginning with spontaneous behaviors (order of tests: elevated plus maze, sucrose neophobia, locomotor activity, social contact, sucrose preference, cold stress defecation) for two weeks, followed by food regulation to 85% body weight over one week and sucrose pellet responding for three weeks. Rats were subjected to one behavioral test at a time and anxiety and appetitive tests occurred prior to drug tests. Rats were then implanted with an indwelling jugular vein catheter and after one week of recovery, behavioral experiments resumed with drug self-administration (acquisition, maintenance, dose response, progressive ratio, and reinstatement). Rats were between 225-250g on arrival and the average weight on the day of the stereotaxic vector injections was 295g (average 293g controls, 296g for shGSK3β). Average weight after spontaneous behaviors and before food restriction prior to sucrose pellet responding was 425g (avg. 424g for controls, 425g for shGSK3β). Average rat weight on the day of catheter implantation was 445g (447g for controls, 445g for shGSK3β). Following drug-self administration, animals were anesthetized, decapitated, and the placement of the vector was verified. See Figure 4.2A for timeline of behavioral testing.

IN VIVO KNOCKDOWN OF GSK3β

In order to knockdown GSK3β *in vivo*, rats were anesthetized with isoflurane (VetEquip, Pleasanton, CA) and injected bilaterally with control vector (AAV-shCTRL) or a novel vector designed to knockdown GSK3β (AAV-shGSK3β) into the NAcSh (1μl/side over 10 min) using stereotaxic coordinates (21-day-old: AP=1.5, L=1.8, V=-5.9; behavior: AP=1.6, L=2.2, V=-6.7, 10° lateral angle). After injection of 1 μl bilaterally, the needles remained in place for 10 additional minutes in order to allow for spread of the vector and to prevent the vector from spreading up the needle track. AAV-shCTRL expresses a non-targeted hairpin, differing only in hairpin sequence (Benzon et al., 2014). Accurate placements were confirmed post-behavioral experiments by anesthesia and decapitation followed by extraction of the brain and visualization of the native enhanced green fluorescent protein (eGFP) fluorescence with a Dual Fluorescent Protein Flashlight and VG2 barrier filter glasses (Nightsea, Bedford, MA) (Anastasio et al., 2014). No animals were excluded based on vector placement. Electrophysiological recordings were made from cells expressing GFP only in the NAc shell region by visual identification with an upright fluorescent microscope.

Construction of Viral Vector Knocking Down GSK3β

The novel AAV2 vector was constructed to decrease GSK3β *in vivo* using methods previously described (Hommel et al., 2003; Benzon et al., 2014). First, target sequences (24nt) were chosen from the coding region of the rat GSK3β mRNA sequence. Optimal sequences contained ~50% cytosine/guanine, little overlap with other mRNAs, and low potential for unwanted secondary structure. Five target sequences were chosen and designed so that the antisense and sense sequences were linked by a miR23 loop. When expressed, the antisense and sense sequences duplex, forming a hairpin structure. Five hairpins were synthesized with XbaI and SapI restriction endonuclease sites,

inserted into an AAV2 plasmid expressing GFP, and verified by sequencing. The mouse U6 promoter expresses the hairpins while the cytomegalovirus promoter expresses eGFP.

IN VITRO VALIDATION OF HAIRPIN

In order to determine which of the five hairpins was most effective *in vitro*, we co-transfected HEK293 cells with a plasmid that overexpresses rat GSK3β and either an shRNA plasmid or a control shRNA plasmid using Lipofectamine 2000 (LifeTechnologies, Grand Island, NY). Forty-eight hours later, the cells were harvested, RNA extracted (RNeasy Minikit, Qiagen) and reverse transcribed to cDNA (SuperScript III First Strand Synthesis: Invitrogen 18080051). Relative knockdown was determined with quantitative real-time PCR (Applied Biosystems, Foster City, CA) on an Applied Biosystems 7500 fast thermocycler with validated primers designed to detect rat GSK3β (forward: TGGCAGCAAGGTAACCACAG; reverse: CGGTTCTTAAATCGCTTGTCCTG). The hairpin plasmid with the highest knockdown *in vitro* was packaged and used for all experiments (5'.CAACTTTACCACTCAAGAACTGTC.3'). AAV was packaged by The University of North Carolina Gene Therapy Core facility. Viral titer was determined using dot blot analysis and ranged from 1 x $10^{10.2}$ to 1 x 10^{13} DRP/ml.

EX VIVO VALIDATION OF PACKAGED VECTOR

In order to validate the knockdown of the AAV-GSK3β vector, the nucleus accumbens from AAV-shCTRL and AAV-shGSK3β injected rats were analyzed via Western blot with previous methods (Zhang et al., 2014). Briefly, the nucleus accumbens were homogenized in a buffer with sucrose, Hepes buffer, sodium fluoride, 10% SDS, and protease and phosphatase inhibitors (Sigma: P-8340, P-2850, P-5726) and the protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Samples were denatured at 95° for 5 min and run on a 10% polyacrylamide gel

(Criterion TGX, Bio-Rad Laboratories, CA, USA) then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was blocked by nonfat dry milk, incubated with mouse anti-GSK3β (1:1000, Cell Signaling) and anti-GAPDH (1:2000, Cell Sig) primary antibodies, then washed with TBST and incubated with fluorescent secondary antibodies (donkey anti-rabbit (680 nm, 1:30000) and donkey anti-mouse (790 nm, 1:15000, Li-Cor Biosciences, NE, USA). Western blots were imaged and protein levels quantified and normalized to GAPDH (Odyssey, Li-Cor Biosciences).

Anxiety-like behavior following GSK3β knockdown

SUCROSE NEOPHOBIA

Three weeks after vector injection, neophobia to a novel taste (sucrose) was assessed in shGSK3β and control rats using methods described previously (Zhang et al., 2014). Rats were separated into new cages at 1600h without access to water, and at 1800h, a 1% w/v sucrose solution in water was placed on each cage for 30 minutes. Sucrose consumption was calculated by the difference in bottle weight from pre-drinking. Rats were returned to their home cages and given a single water bottle with the same 1% w/v sucrose solution in water for 72hrs, ending 48hrs prior to the sucrose preference test.

COLD STRESS-INDUCED DEFECATION

Anxiety-like behavior was also assessed by measuring the amount of defecation in response to a mild stressor (cold). Polycarbonate mouse cages (33x17x13cm) were pre-chilled on ice for 10 min or until the temperature was approximately 12°C. Rats were placed into the cages on ice (one rat per cage) and the number of fecal boli produced were recorded every 5 min for 30 min. After 30 min, rats were returned to their home cages.

ELEVATED PLUS MAZE

Anxiety-like behavior was examined by placing animals on an elevated maze with two open arms and two closed arms measuring 12 x 50 cm, 75 cm above the floor for 5 min (~60 lux open arms, ~20 lux closed arms; Med Associates Inc., VT, USA). The amount of time spent on the open arms and closed arms along with the number of open arm entrances and closed arm entrances were analyzed by photobeam breaks using Med-PC software (Zhang et al., 2014).

Depression-like behavior following GSK3β knockdown

SUCROSE PREFERENCE

Depression-like behavior was assessed by determining preference for a 1% w/v sucrose solution over water. Rats were separated and placed into new cages without access to water at 1600h for 2 hrs. At 1800h, a bottle of water was placed in the normal water position and a bottle with sucrose (1% w/v) was placed ~10cm away. Rats were allowed to drink from either bottle for 30 minutes then returned to their home cages and the bottles were weighed. Percent sucrose consumed was determined by dividing the change in weight of the sucrose bottle divided by the change in weight of both the sucrose and the water bottles.

SOCIAL CONTACT

Differences in social behavior were assessed by measuring grooming behavior after 24hrs of separation. Rats were separated in new cages for the 24hrs immediately prior to the social contact test. Control and shGSK3β rats were placed in a novel environment (plastic container, 45x40x45cm) with a 2-inch layer of regular corncob bedding with their original cage-mate (controls with controls and knockdown with knockdown). Behavior was video recorded for 30 min. Rats were returned to their home

cages after 30 min. The time spent grooming each other was measured for each pair by a researcher blinded to the conditions (Zhang et al., 2014).

Cocaine Self-Administration following GSK3β knockdown

Intravenous catheter implantation

Rats were anesthetized with ketamine (100 mg/kg IP) and xylazine (10 mg/kg IP) and a Silastic catheter was inserted into the jugular vein exiting the animal's back (Green et al., 2008; Zhang et al., 2014). To promote catheter patency and prevent infection, catheters were infused daily with 0.1 ml of sterile saline solution with heparin (30.0U/ml), penicillin G potassium (250,000U/ml), and streptokinase (800U/ml). Animals recovered for 7 days before beginning cocaine self-administration (SA). One animal lost catheter patency during SA and did not complete all SA tests.

Acquisition

Prior to catheter implantation, rats were food restricted to 85% body weight over 7 days then placed in operant chambers (30x24x21cm, Med-Associates, St. Albans, VT) with one active lever, one inactive lever, two cue lights, a food hopper/dispenser, and a house light. Rats were trained to lever press for sucrose pellets on fixed ratio (FR) 1-FR5 for 15 min per day and trained on extinction and progressive ratio schedules of reinforcement (Zhang et al., 2014). After recovery from catheter surgery, rats were allowed to self-administer cocaine dissolved in saline (0.2 mg/kg/infusion, Research Triangle Institute, NC) on an FR1 schedule for 2hrs/day for 7 days. Infusions were signaled by illumination of two cue lights above the active and inactive levers for 20s, which also signaled a timeout period during which no further infusions could be obtained. Infusions were delivered intravenously in a volume of 0.1 ml over 5.8s upon depression of the active lever (left).

MAINTENANCE

After animals acquired the task, maintenance responding was assessed at a higher dose of cocaine (0.5 mg/kg/inf) for 2hrs/day for 5 days on an FR1 schedule.

FIXED RATIO DOSE RESPONSE

Prior to dose response, rats underwent extinction training for 3 days where cocaine (0.5 mg/kg/inf) was available for 1hr followed by 3hrs where lever presses resulted in illumination of cue lights but no drug infusions. Rats then self-administered cocaine at 8 different doses (0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.0075, 0.00325 mg/kg/inf) in descending order for 30 min per dose for a total session time of 4hrs on an FR1 schedule of reinforcement for five days.

PROGRESSIVE RATIO

Rats self-administered cocaine on a progressive ratio schedule of reinforcement (1, 2, 4, 6, 9, 12,15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 603, 737, 901) at a dose of 0.5 mg/kg/inf for 3 sessions and then 0.125 mg/kg/inf for 3 sessions. The program ended and rats were removed from the chamber when they did not receive an infusion for more than 1 hour or after 8hrs total.

COCAINE-INDUCED REINSTATEMENT

Rats self-administered cocaine for 1 session (0.5 mg/kg/inf; 2 hrs; FR1) before a within-session reinstatement procedure began. Rats received 0.5 mg/kg/inf on an FR1 schedule for 1hr followed by 3hrs of extinction (with contingent cue light). Next, they received an IV infusion of cocaine of one of five doses (0, 0.0625, 0.125, 0.25, 0.5 mg/kg) in a semi-random order for each rat across the 5 sessions of reinstatement (pattern of doses was randomly assigned for 10 animals and applied to both groups). Cocaine-induced reinstatement responding was assessed for 3h following the single IV infusion

where the animals again received cocaine cues but no infusions as with extinction. Reinstatement sessions were followed by 2 intervening days of high dose (0.5 mg/kg/inf) cocaine on an FR1 schedule for 2h to maintain a high level of responding (Green et al., 2010).

Electrophysiology following GSK3β knockdown

SLICE PREPARATION

Acute coronal slices containing the NAc were prepared from control and shGSK3β rats 21-30 days following surgery. Rats were decapitated, brains were dissected and 300μm coronal slices containing the NAc were prepared with a vibratome (Leica Biosystems, Buffalo Grove, IL) in an iced sucrose-based artificial cerebral spinal fluid (aCSF), consisting of the following (in mM): 56 NaCl, 100 Sucrose, 2.5 KCl, 20 glucose, 5 MgCl2, 1 CaCl2, 30 NaHCO3, and 1.25 NaH2PO4, osmolarity 300–310 and continuously oxygenized and equilibrated to pH 7.4 with a mixture of 95% O2/5% CO2. Slices were transferred to an incubation chamber with standard aCSF consisting of the following (in mM): 130 NaCl, 3.5 KCl, 10 glucose, 1.5 MgCl2, 1.4 CaCl2, 23 NaHCO3, and 1.25 NaH2PO4, osmolarity 300–310, oxygenated and equilibrated to pH 7.4 with a mixture of 95% O2/5% CO2 at 31°C.

LOOSE-PATCH RECORDING AND DATA ANALYSIS

After 1–2hrs of recovery, acute brain slices were placed in a submerged recording chamber on the stage of an upright microscope (Axioskop2 FS plus; Zeiss). Slices were continuously perfused at room temperature with standard aCSF (~2ml/min) and equilibrated for 15-20 minutes prior to recordings. Loose-patch recordings were obtained from visually identified GFP positive neurons expressing AAV shRNA against GSK3β or scramble shRNA neurons in the NAcSh. Recording pipettes (3–4 MOhm) were fabricated from borosilicate glass (WPI) using a two-step vertical puller PC-10

(Narishige), and filled with standard aCSF. Loose-patch somatic recordings of spontaneously active neurons of relative large soma size corresponding to what has been previously characterized as tonically active neurons (Pisani et al., 2007) were performed using a MultiClamp 700B (Molecular Devices), low-pass filtered at 2.2 kHz, and sampled at 20 kHz using a Digidata 1322A analog-to-digital interface and pClamp9 acquisition software (Molecular Devices). The seal resistance for loose-patch recordings was typically 50–100 MΩ.

WHOLE CELL PATCH CLAMP RECORDING AND DATA ANALYSIS

Whole-cell patch-clamp experiments were performed using Axopatch 200B and Multiclamp 700B amplifiers. Somatic recordings in standard aCSF from visually identified TANs were performed with pipettes (resistance of 3–5 MΩ) filled with an internal solution containing (in mM): 145 K-gluconate, 2 MgCl2, 0.1 EGTA, 2 Na2ATP, and 10 HEPES (pH 7.2 with KOH; 290 mOsm). After seal formation and cell membrane rupture, TANs were held in voltage-clamp mode for 1-2 minutes with subsequent switch to current clamp mode to assess neuronal activity. Data acquisition and stimulation were performed with a Digidata 1322A Series interface and pClamp 9 software (Molecular Devices, Sunnyvale, CA). Data were filtered at 2 kHz, digitized at 20 kHz. Neuronal intrinsic excitability was assessed by measuring evoked action potentials with a range of current injections of constant increment (800 msec current square pulses with 10 pA increment). Action potential (AP) current threshold (Itrh) was defined as the current step at which at least one spike was induced. AP voltage threshold (Vtrh) was defined as the voltage at which the first-order derivative of the rising phase of the AP exceeded 10 mV/ms (Nenov et al., 2015). Maximum rise and decay of APs were defined as maximal derivate value (dV/dt) of the depolarizing and repolarizing phases of the AP, respectively. Spontaneous action potential firing from TANs was assessed in current clamp mode at resting membrane potential. To analyze the sag of TANs, cells were set to membrane

potential of -70 mV with current injection. The range of current steps from -120 pA to +20 pA with 20 pA increment (500 ms duration) was applied to estimate sag amplitude. All electrophysiology data were analyzed with pCLAMP 10 and GraphPad Prism 6 software.

STATISTICAL ANALYSIS

Statistical significance was set at $p<0.05$ and all data are expressed as mean ±SEM. Students t-test was used to compare means for single-factor analyses. The Welch-Satterthwaite method was used to adjust df in cases where there was a violation of the homogeneity of variance. The Mann-Whitney test was used for the cold stress-induced defecation, as these data are not normally distributed. Two-way repeated measures ANOVAs were used to compare differences among conditions for remaining experiments using SPSS and GraphPad Prism 6 software.

RESULTS

Vector knockdown validation

The hairpin sequence was first validated in vitro by co-transfecting HEK293 cells with a plasmid expressing GSK3β and either the control hairpin plasmid or each of the GSK3β hairpin plasmids and comparing the amount of GSK3β mRNA in the cells. The chosen shRNA construct reduced the amount of GSK3β mRNA by > 90% (Figure 4.1A). AAV-shGSK3β was further validated *in vivo* by injecting AAV-shGSK3β or AAV-shCTRL into the NAcSh and comparing the amount of GSK3β protein expression via Western blot analysis. AAV-shGSK3β decreased the GSK3β protein level in the NAc by 35% (Figure 4.1B, normalized to GAPDH). This *in vivo* knockdown level is a similar level as a previous AAV RNA interference study (Hommel et al., 2003). The nature of gross dissection is that the majority of the cells present are non-neuronal and not

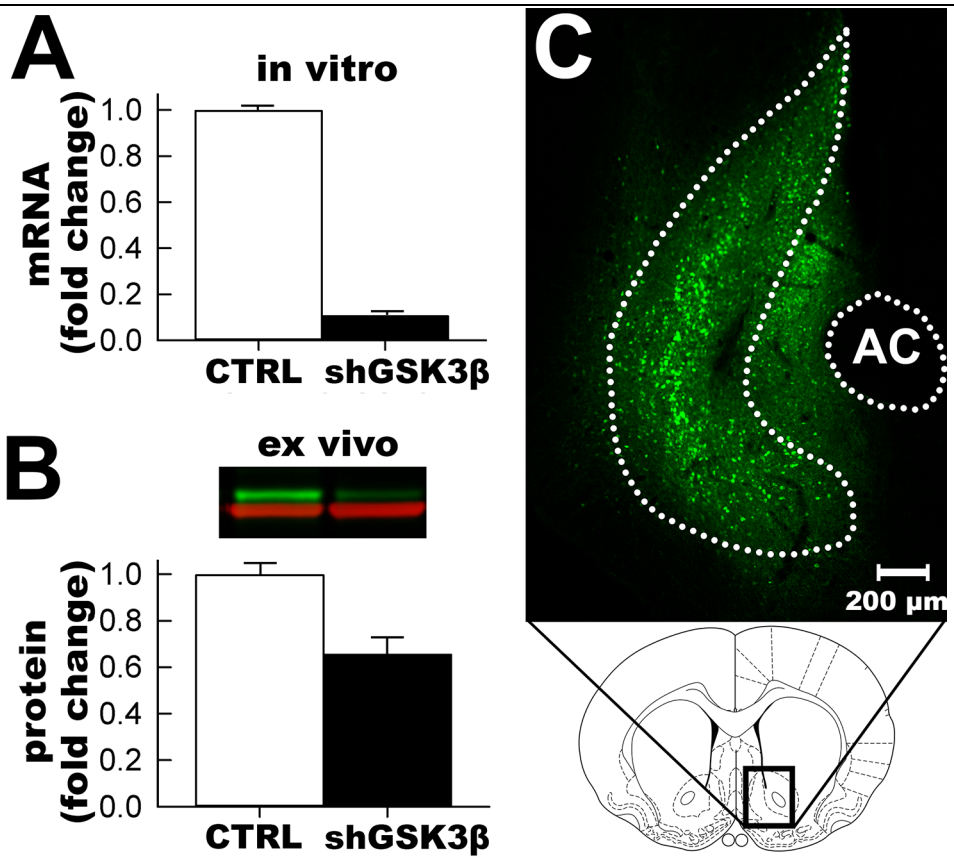

Figure 4.1 GSK3β shRNA vector validation

A. *In vitro* validation of mRNA knockdown in HEK293 cells. B. Quantification of *in vivo* validation of knockdown of GSK3β protein (green channel) normalized to GAPDH (red channel) in the NAcSh via Western blot (inset). C. Representative placement of viral vector (eGFP immunofluorescence) in the NAcSh (NAcSh outlined and AC=anterior commissure).

transduced by the vector, so 35% knockdown is expected and represents *in vivo* validation of the vector.

Vector placement validation

Accurate placement of the vectors was confirmed in all rats after the behavioral tests by extracting the brains and visualizing GFP fluorescence with a DFP flashlight while wearing VG2 barrier filter glasses. All rats had GFP expression in the left and right accumbens shell regions and spread of the vector was roughly spherical with a diameter of 1mm and generally between 2.2mm to 0.7mm anterior from bregma, which is expected

with our stereotaxic coordinates. Although some GFP could be seen outside the NAcSh, the majority of GFP positive cells were in the accumbens shell, consistent with our previous vector studies (Green et al., 2006; Green et al., 2008; Wallace et al., 2008; Green et al., 2010). No rats were excluded from analysis based on vector placement (Figure 4.1C). Electrophysiological recordings were confined to GFP positive cells in the nucleus accumbens shell region via visual identification.

Knockdown of GSK3β in the NAcSh produces anxiolytic-like behavior

The effect of reduced GSK3β in the NAcSh on anxiety-like behavior was tested with three assays. We found that knockdown animals consumed more sucrose than controls (t (18) = −2.1, p = 0.048) when unfamiliar with the taste of sucrose (Figure 4.2B). In addition, knockdown rats defecated less compared to controls in a mild-stress environment (i.e. cold; Figure 4.2C, U = 29, p = 0.049). We found no significant differences in open/closed arm exploration in the elevated plus maze due to high variance and no differences in open/closed arm entrances (Figure 4.3 A-B). Although we found no effect in the EPM test, the sucrose neophobia and cold stress defecation tests both indicate that knockdown of GSK3β in the NAcSh produces anxiolytic-like behavior.

Knockdown of GSK3β in the NAcSh increases depression-like behavior

Although GSK3β knockdown rats consumed more sucrose in the neophobia test (i.e. anxiety-related), we found that these same rats preferred the same sucrose concentration less than controls when familiar with the taste (i.e. depression-related; Figure 4.2D, t (19) = 2.1, p = 0.046). Modeling social withdrawal, we found that shGSK3β rats groomed each other less than controls after a brief separation (Figure 4.2E, t (4.5) = 3, p = 0.035). The assumption of homogeneity of variance in the social contact test was violated so the t-test degrees of freedom were adjusted to correct for this. It is important to note that this difference in grooming behavior was not due to differences in

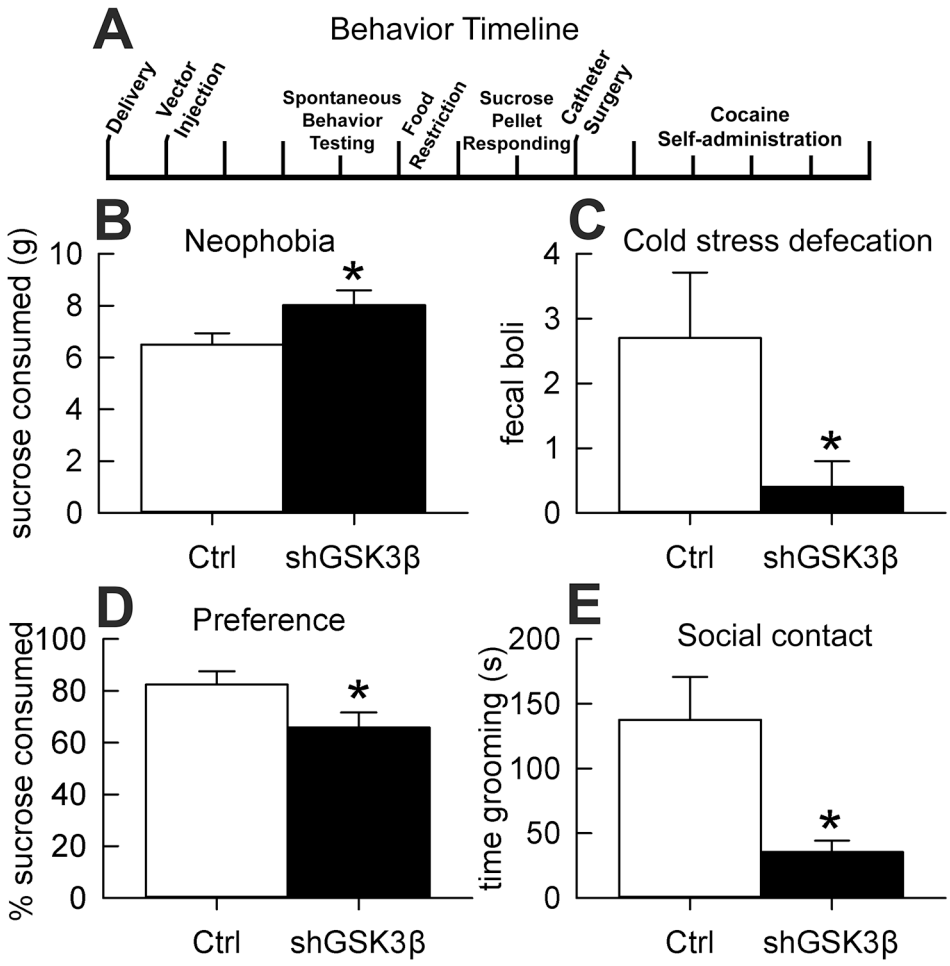

Figure 4.2 Effect of GSK3β knockdown in NAcSh on anxiety- and depression-like behavior

A. Timeline of behavioral experiments. B. Sucrose neophobia. Mean (±SEM) difference in 1% sucrose solution consumed for 30 min (n=10). C. Cold stress-induced defecation test. Mean (±SEM) fecal boli produced after 15 minutes of cold stress (n=10). D. Sucrose preference. Mean (±SEM) percent sucrose consumed (n=10-11). E. Social grooming behavior. Mean (±SEM) seconds spent grooming cage-mate across 30 min session (n=5). *Significant difference from controls, $p < 0.05$.

general locomotor activity, as spontaneous locomotor activity did not differ between knockdown and control rats (Figure 4.4). Sucrose preference is a model of anhedonia and social contact is a model of social withdrawal, therefore these results suggest knockdown of GSK3β in rat NAcSh increases depression-like behavior.

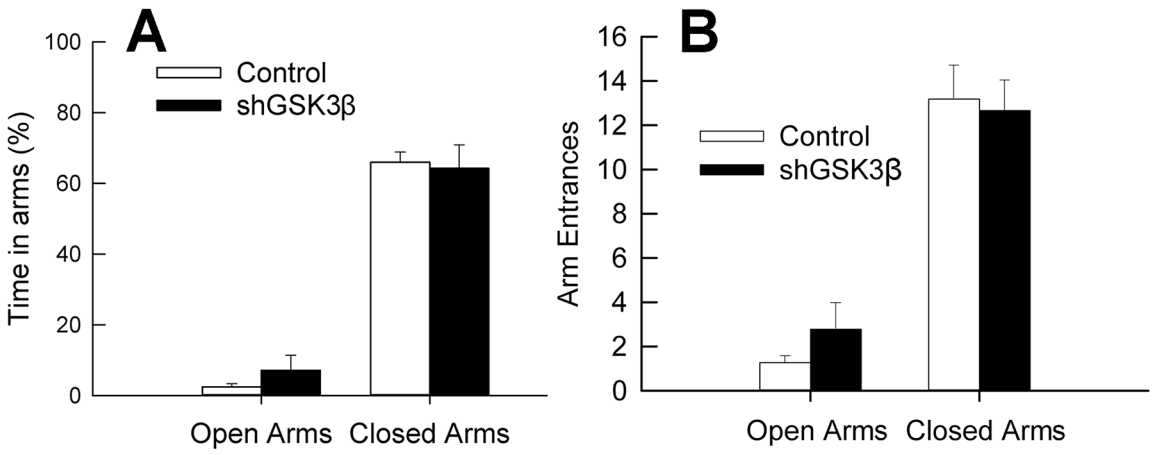

Figure 4.3 Elevated plus maze

Comparison of open arm exploration time and closed arm exploration time (A) and open arm entrances and closed arm entrances (B) between control vector injected animals and GSK3β knockdown vector injected animals. $p > 0.05$.

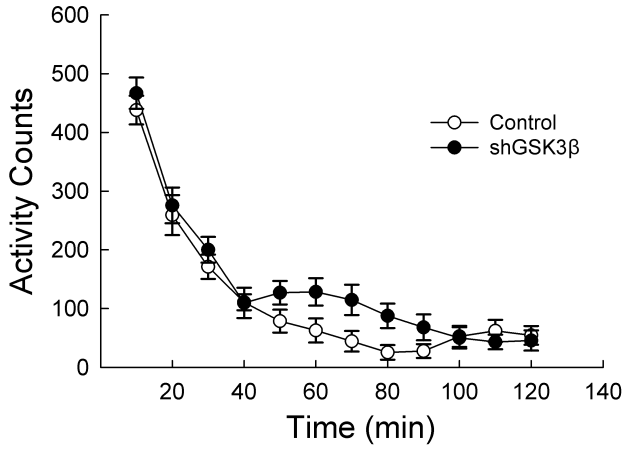

Figure 4.4 Spontaneous locomotor activity

Locomotor activity counts between control vector injected animals and GSK3β knockdown vector injected animals. $p > 0.05$.

Knockdown of GSK3β in the NAcSh increases addiction-related behavior

Cocaine-taking and seeking behavior was assessed in GSK3β knockdown animals and controls using the intravenous cocaine self-administration paradigm. Prior to cocaine SA, shGSK3β and control rats were assessed for their ability to learn an operant task and

we found no differences in operant responding for sucrose pellets (data not shown). However, we did find differences in cocaine-taking and seeking behavior. For acquisition, Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated, $\chi^2(20) = 78.4$, $p < 0.001$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε=0.48). The results show a main effect of session during acquisition of cocaine self-administration ($F(1,2.9) = 7.05$, $p < 0.001$) and a trend toward increased cocaine taking in shGSK3β rats during acquisition

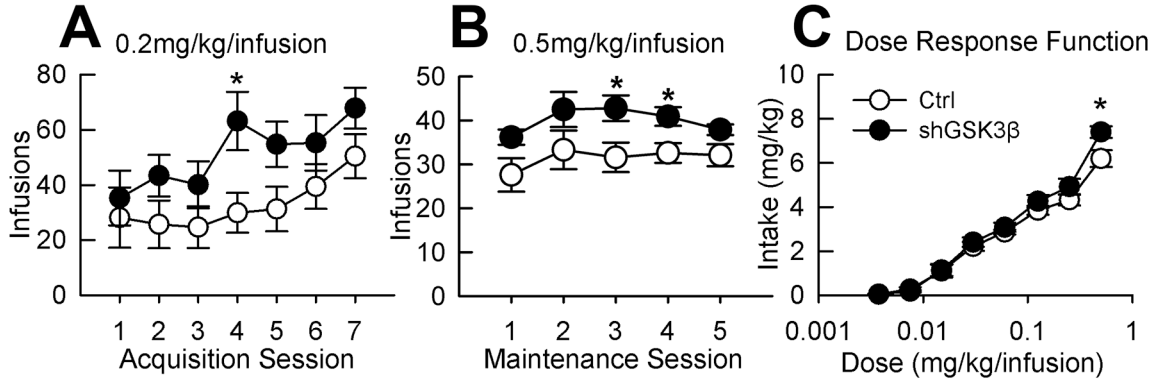

Figure 4.5 Knockdown of GSK3β in the NAcSh increases cocaine self-administration

A. Effect on acquisition of cocaine self-administration. Mean (±SEM) number of infusions of cocaine in control or shGSK3β rats self-administering 0.2mg/kg/inf cocaine. B. Effect on maintenance of cocaine self-administration. Mean (±SEM) number of infusions of cocaine in control or shGSK3β rats of 0.5mg/kg/inf cocaine for 2 hr/session. C. Dose response function of self-administration. Mean (±SEM) intake (mg/kg) of cocaine at each dose (30 min ea). (n=10-11) *Significant difference from controls, $p < 0.05$.

(Figure 4.5A, $F(1,19) = 3.4$, $p = 0.082$). There was no significant interaction for acquisition of cocaine self-administration (Figure 4.5A, $F(1,2.9) = 1.15$, $p = 0.33$). For maintenance responding Mauchly's Test of Sphericity indicated the assumption of sphericity had been violated, $\chi^2(9)=21.7$, $p < 0.01$, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.66$). There was a significant increase in maintenance responding at 0.5 mg/kg/inf in shGSK3β rats

compared to control rats (Figure 4.5B, F (1,18) = 6.3, p = 0.021) with a main effect of session (F (1,2.62) = 2.95, p = 0.049). There was no significant interaction effect for maintenance responding (F (1,2.62) = 0.48, p = 0.672). We found no significant differences between shGSK3β and control rats in extinction responding or reinstatement responding (data not shown, p>0.05). For cocaine dose response, again Mauchly's Test of Sphericity indicated the assumption of sphericity had been violated $\chi^2(27)=83.2$, p < 0.001, therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = 0.42). There was no main effect of group (F (1,18) = 2.06, p = 0.168), however controls and shGSK3β rats both produced dose response functions for cocaine with a significant main effect of dose (F (1,2.96) = 340, p < 0.001) and there was a significant dose x group interaction (F (1,2.96) = 3.7, p < 0.018). The nature of this interaction was shGSK3β rats show increased intake at the highest dose. Overall, our cocaine self-administration results show that knockdown of GSK3β in the NAcSh altered addiction-related behavior, increasing maintenance responding, a trend toward increased acquisition, and an interaction of dose and group for dose response.

Knockdown of GSK3β in the NAcSh reduces spontaneous neuronal firing

To determine whether GSK3β knockdown correlated with functional outcomes of neuronal activity, AAV-shGSK3β or control vector was stereotaxically injected into the NAcSh and acute brain slices prepared 3-4 weeks later. Loose-patch recordings of visually identified, eGFP-expressing neurons revealed that GSK3β knockdown led to a significant decrease in spontaneous firing rate compared to TANs from control animals (Figure 4.6A-B, t (36) = 2.3, p = 0.025) and an increase in the spike half-width (Figure 4.6C-D; t (35) = 3.022, p = 0.0047).

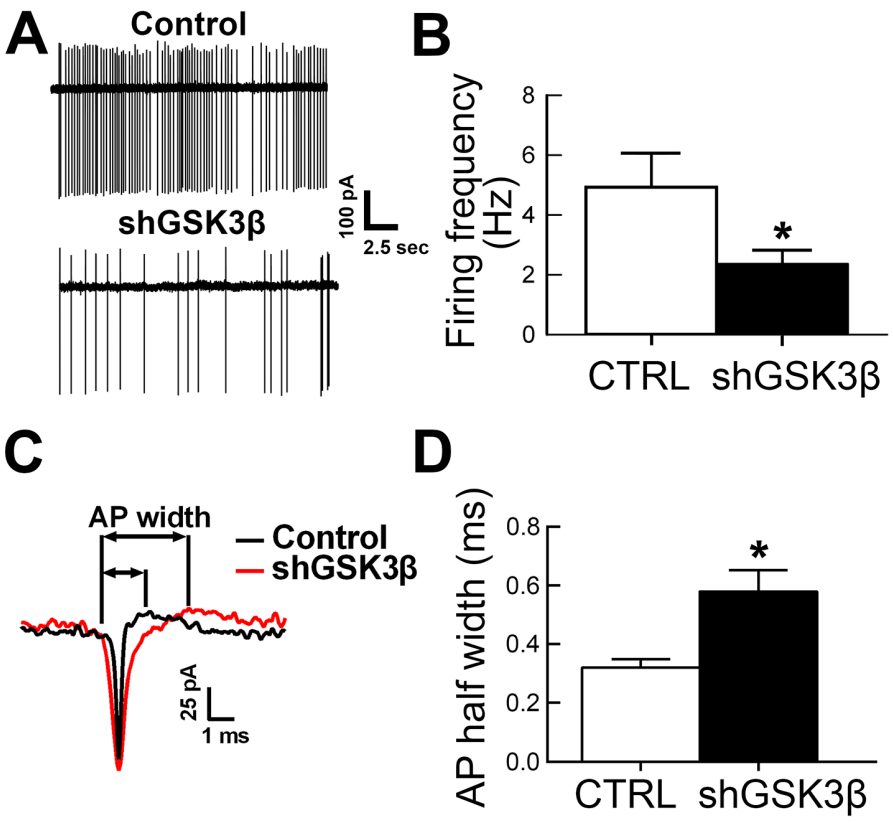

Figure 4.6. GSK3β knockdown in NAcSh reduces tonic neuronal activity and increases action potential half width in NAcSh neurons

A. Representative traces of tonic activity in GSK3β knockdown compared to control NAcSh neurons. B. Summary bar graph of loose-patch recorded tonic activity, control n=16 cells, shGSK3β n=22 cells (mean±SEM); control n=2 rats, shGSK3β n=2. C. Representative traces of action potential width in GSK3β knockdown compared to control NAcSh neurons. D. Summary bar graph of AP half widths, control n=16 cells, shGSK3β n=21 cells (mean±SEM); control n=2 rats, shGSK3β n=2. *$p < 0.05$. Loose patch-clamp recordings were collected by Miroslav N. Nenov, Ph.D. and Federico Scala, Ph.D. in the laboratory of Fernanda Laezza, M.D., Ph.D. on animals injected with AAV-shCTRL or AAV-shGSK3β by Elizabeth J. Crofton.

Knockdown of GSK3β in the NAcSh altered intrinsic excitability of TANs

To determine whether reduced spontaneous firing in loose-patch correlated with changes in intrinsic excitability, whole-cell patch clamp recordings of TANs in both control vector and GSK3β knockdown vector conditions were performed. Representative traces of spontaneous firing recorded at resting membrane potential are shown for control vector

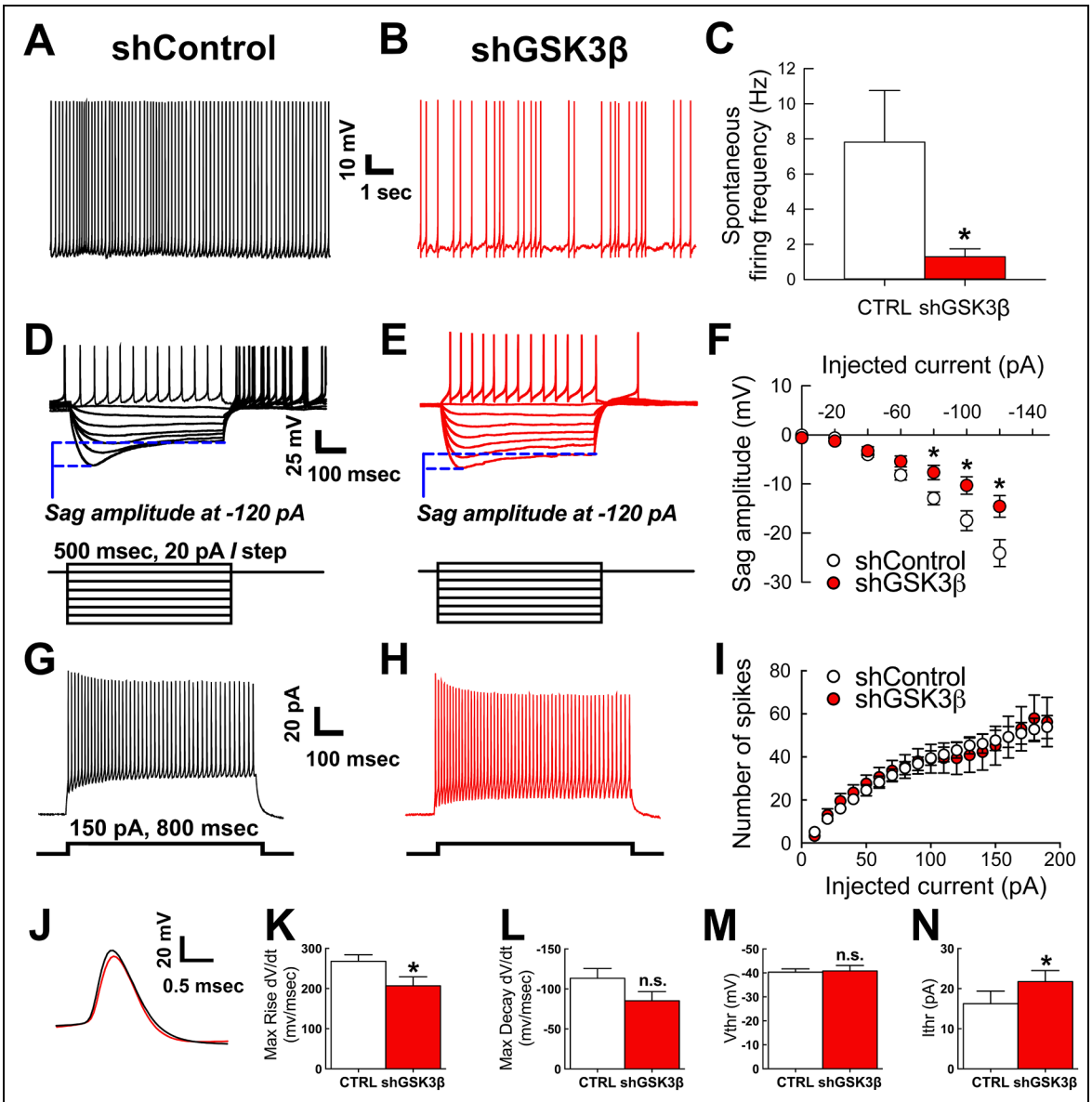

Figure 4.7. Whole cell patch clamp recordings of neurons with spontaneous activity in both control vector and GSK3β knockdown vector show tonically active interneuron (TAN) phenotype and GSK3β knockdown in NAcSh reduces spontaneous neuronal activity in identified tonically active interneurons (TANs)

A-B. Representative traces of spontaneous firing at resting membrane potential recorded from shControl (A) and shGSK3β (B) neurons in whole-cell patch clamp. C. Summary bar graph of action potential firing frequency recorded in whole cell, control n=13 cells and shGSK3β n=15 cells (mean±SEM). Control n=6 rats, shGSK3β n=8 rats. D-E. Passive properties of same neurons as A-B showing hyperpolarizing sag and action potential rebound in control (D) and shGSK3β knockdown (E) with injected current (500 msec, 20 pA/step). F. Summary graph of sag amplitude (mV) at different injected current (pA) showing reduced sag amplitude in GSK3β knockdown (n=18 cells) versus control (n=13 cells) (mean±SEM). Control n=6 rats, shGSK3β n=8 rats. G. and H.

Representative traces from control vector (G) and shGSK3β vector (H) transduced TANs showing trains of evoked action potentials at 800 msec and 150 pA current step injection. I. Input-output curve of evoked AP trains in response to repetitive current steps of constant increment. J. Action potential waveforms evoked at the current threshold are illustrated for control (black line) or shGSK3β vector (red line) transduced TANs. K. Bar graph showing reduced maximum rise (dV/dt, mV/msec) in GSK3β knockdown (n=17 cells) versus control (n=16 cells) (mean±SEM); control n=6 rats, shGSK3β n=8 rats. L. Bar graph showing no significant difference in action potential maximum decay (dV/dt, mV/msec) in GSK3β knockdown (n=17 cells) versus control (n=16 cells) (mean±SEM); control n=6 rats, shGSK3β n=8 rats. M. Bar graph showing no difference in the action potential voltage threshold (Vtrh, mV) in GSK3β knockdown (n=17 cells) versus control (n=16 cells) (mean±SEM); control n=6 rats, shGSK3β n=8 rats. N. Bar graph showing reduced current threshold (Itrh, pA) in GSK3β knockdown (n=17 cells) versus control (n=16 cells) (mean±SEM); control n=6 rats, shGSK3β n=8 rats. $*p < 0.05$. Whole cell patch-clamp recordings were collected by Miroslav N. Nenov, Ph.D. and Federico Scala, Ph.D. in the laboratory of Fernanda Laezza, M.D., Ph.D. on animals injected with AAV-shCTRL or AAV-shGSK3β by Elizabeth J. Crofton.

in Figure 4.7A and GSK3β knockdown vector in Figure 4.7B. The spontaneous firing frequency was significantly lower in the cells transduced with the knockdown vector (t (26) = 2.357, p = 0.026, Figure 4.7C). When TANs are hyperpolarized to negative voltages, a typical sag potential is observed as a result of activation of HCN-mediated Ih current (Bennett and Wilson, 1999; Wilson, 2005; Deng et al., 2007; Pisani et al., 2007). Accordingly, upon membrane hyperpolarization, typical depolarizing sag potentials in response to different current injections were observed in TANs from both control and the knockdown groups (inset, 500 msec, 20pA/step Figure 4.7D-E). Notably, though, GSK3β knockdown vector-transduced neurons exhibited a significant reduction in the amplitude of this hyperpolarization-induced sag at −80 pA (t (29.99) = 2.704, p = 0.011), −100 pA (t (26.5) = 2.670, p = 0.013), and −120 pA (t (26.66) = 2.710, p = 0.012). Intrinsic excitability of GSK3β knockdown transduced TANs and controls was further characterized by analyzing the input-output curve corresponding to the neuron firing frequency in response to incremental steps of depolarizing current injections (Figure 4.7G-I). As illustrated in Figure 4.7I, no changes in firing frequency were found in the two groups across all tested current steps. However, additional analysis revealed

significant changes in the action potential (AP) kinetics and threshold induced by GSK3β knockdown (Figure 4.7J-N). In GSK3β knockdown transduced TANs, the AP maximum rise was reduced compared to the control vector (t (31)=2.167, p = 0.038; Figure 4.7K) while no significant changes were found in the AP maximum decay (Figure 4.7L). Furthermore, AP current threshold was significantly increased in the GSK3β knockdown group (U (220,341) = 84, p = 0.0423; Figure 4.7N), while no changes were found in AP voltage threshold (Figure 4.7M). These results indicate that knockdown of GSK3β in the NAcSh alters intrinsic excitability of TANs, likely contributing to reduced spontaneous firing.

DISCUSSION

This study found that GSK3β in the NAcSh of rats modulates addiction-, depression-, and anxiety-related behavior and also causes a reduction in spontaneous activity of TANs attributable to changes in intrinsic excitability. The behavioral phenotype mimics isolation or a susceptible phenotype.

Knockdown of GSK3β in the NAcSh specifically results in decreased anxiety-like behavior in the sucrose neophobia and cold-stress induced defecation tests but no change in behavior in the elevated plus maze, likely due to the high within-group variance inherent in this behavior. Additionally, knockdown of GSK3β increased depression-like behavior in the sucrose preference and social contact tests and increased drug taking behavior in cocaine self-administration maintenance responding, with a trend for an increase in acquisition. However, there were no differences in drug seeking behavior in extinction, progressive ratio, or cocaine-induced reinstatement tests.

Although depression and anxiety are often (but certainly not always) comorbid in humans (Kessler et al., 2005; Pettinati et al., 2013), the opposite modulation of depression and anxiety in the current studies is perfectly consistent with previous manipulation of the NAcSh, (Green et al., 2006; Green et al., 2008; Green et al., 2010) as

is the congruent behavioral phenotypes for depression and addiction (Hikida et al., 2001; Green et al., 2010; Larson et al., 2011; Warner-Schmidt et al., 2012). This could be idiosyncratic to the NAcSh, or to rats in general; regardless, the current behavioral phenotypes (decreased anxiety-like, but increased depression- and addiction-like behavior) are perfectly consistent with what we understand about rat NAcSh.

In behaving animals, tonically active cholinergic interneurons modulate release of dopamine in the accumbens and have been shown to play an important role in drug-related behaviors (Green et al., 2001; Berlanga et al., 2003; Pisani et al., 2007; Witten et al., 2010; Cachope et al., 2012). Consistent with our current behavioral and electrophysiological results, it has been reported that increased depression-like behavior and addiction-related behavior in the NAc can be induced by ablation of tonically active cholinergic interneurons (Hikida et al., 2001; Warner-Schmidt et al., 2012).

Tonically active neurons (TANs) are critical interneurons in the NAcSh previously shown to be important for reward processing (Hikida et al., 2001; Berlanga et al., 2003; Apicella, 2007; Witten et al., 2010; Cachope et al., 2012). They receive inputs from the cortex (glutamatergic), substantia nigra and VTA (dopaminergic), and from medium spiny neurons (GABAergic) and synapse mainly onto MSNs and other tonically active neurons (Lenz and Lobo, 2013). Although TANs are a relatively small percentage of the total neuronal population in the accumbens, they exert a powerful modulation of the NAc circuit activity through highly divergent and dense axonal projections (Lim et al., 2014) and are capable of altering cocaine conditioned place preference behavior in freely moving animals (Witten et al., 2010). In addition, TANs in the NAcSh are activated by cocaine self-administration (Berlanga et al., 2003). The regulation of TANs appears to be brain sub-region specific, for optogenetic activation of dopamine neurons causes TANs in the NAcSh to fire a specific burst-pause pattern, but other regions like the nucleus accumbens core and the dorsal striatum do not consistently show this pattern in response to dopaminergic activation (Chuhma et al., 2014). Additionally, optogenetic

activation of TANs in the accumbens stimulates release of dopamine (Cachope et al., 2012) and reduced number of TANs causes hyperresponsiveness of the dopamine system and increased sensitivity to cocaine (Hikida et al., 2001; Laplante et al., 2011). This suggests that reduced TAN firing in the NAcSh is integral to depression-like behavior and cocaine self-administration, but because the GSK3β shRNA vector also transduced MSNs, further work is needed to determine a direct causal link between GSK3β in TANs and the behavioral results.

GSK3β has not yet been implicated in the reward processing of TANs, however, GSK3β has been suggested to be downstream of dopamine receptors, specifically the dopamine D2 receptor DRD2 (Beaulieu et al., 2011; Sutton and Rushlow, 2012; Urs et al., 2012). Dopamine receptors typically signal through G proteins, however they also have G protein independent signaling through β-arrestin, which is upstream of AKT and GSK3. There is some evidence that GSK3β even forms a protein complex with the dopamine D2 receptor (Sutton and Rushlow, 2012). Previous work has shown that D2 receptors in NAcSh are important for the reinstatement of cocaine seeking (Anderson et al., 2006). D2 receptors are localized on cholinergic interneurons in the NAcSh (Alcantara et al., 2003) and D2 receptor agonists and antagonists but not D1 receptor compounds alter the activity of TANs (Deng et al., 2007). Therefore, knockdown of GSK3β may be influencing dopamine signals, contributing to TAN firing reduction.

The previous paragraph addresses mechanisms upstream of GSK3β, but the question remains how GSK3β can alter neuronal activity of TANs. The electrophysiological phenotypes reported in this study are consistent with a role of GSK3β in modulating spontaneous neuronal firing in TANs through effects on voltage-gated ion channels that are independent from the network activity.

TANs recorded *in vivo* respond to sensory stimuli associated with learned behavior with a transient depression of tonic firing (Wilson et al., 1990; Reynolds et al., 2004b; Apicella, 2007). Unlike in other neurons, firing in TANs is largely controlled by a

combination of intrinsic voltage-gated conductances including Ih, Na+ persistent currents, and hyperpolarization-activated K+ channels that drive oscillations of the membrane potential (Wilson, 2005). Our whole-cell patch clamp analysis indicates that knockdown of GSK3β suppresses hyperpolarization-induced sag potentials and decreases action potential max rise while increasing AP threshold, suggesting a role of the Ih pacemaker current in establishing these phenotypes (Ko et al., 2016). TANs firing is controlled by the neuron intrinsic properties that usually dominate over stimuli arising from synaptic inputs (Wilson, 2005). Thus, it is conceivable that the suppression of spontaneous firing induced by GSK3β knockdown in the intact NAcSh circuit (Figure 4.6 and Figure 4.7A-C) is driven by autonomous changes in the neuron intrinsic properties rather than being network-driven.

Previous results from the hippocampus indicate that GSK3β regulates protein-protein interactions within the voltage-gated sodium channel (Nav) complex (Shavkunov et al., 2013) and investigation of Nav1.2 specifically found that GSK3β phosphorylates this channel, altering its functional properties (James et al., 2015). The lack of changes in maximum firing frequency of evoked action potentials in TANs expressing AAV-shGSK3β (Figure 4.7I) argues against a major involvement of Nav channels in mediating the observed phenotypes. However, which specific channels are GSK3β substrates in TANs should be addressed with future voltage-clamp studies.

The AKT/GSK3 intracellular signaling cascade is generally implicated in the etiology of several psychiatric disorders with relevance to the nucleus accumbens (Beaulieu et al., 2009). Dysregulation of GSK3β is thought to play a role in susceptibility to depression and bipolar disorder (Jope, 2011) and is associated with other diseases such as schizophrenia, and even Alzheimer's disease (Kozlovsky et al., 2001; Emamian et al., 2004; Balaraman et al., 2006; Hooper et al., 2008). Efforts to globally knock out GSK3β in mice have proven embryonically lethal (Hoeflich et al., 2000), but heterozygous knockout mice survive and provide insights into the importance of GSK3β. These mice

show reductions in depression-like behavior similar to the behavioral results seen with lithium, an inhibitor of GSK3β (O'Brien et al., 2004). However, lithium itself is not just an inhibitor of GSK3β but has other mechanisms of action and it is likely that the behavioral effects of the heterozygous knockout mice are a result of GSK3β action during development or in a different brain region, underscoring the utility of region-specific knockdown of GSK3β in adult animals.

Wilkinson et al., 2011 found that after social defeat stress, mice injected with a herpes simplex virus (HSV) overexpressing GSK3β in the nucleus accumbens showed a depression-like phenotype. Whereas the current study found that with little prior stress exposure, rats with decreased GSK3β in the nucleus accumbens with an AAV vector also show a depression-like phenotype, indicating modulatory effects of GSK3β under various levels of stress exposure. These seemingly disparate findings show GSK3β has important modulatory roles that can be adaptive or maladaptive even in the same brain region depending on stress exposure. We have previously hypothesized that the robust behavioral phenotypes resulting from environmental enrichment (i.e. decreases in depression-like behavior and decreases in cocaine self-administration (Green et al., 2010)) result from repeated mild stress exposure in the enriched animals and a lack of stress exposure in isolated animals (Crofton et al., 2015). Chronic mild stress in the case of environmental enrichment is adaptive whereas a severe stressor, such as social defeat, is maladaptive (Crofton et al., 2015). Therefore, our behavioral findings with knockdown of GSK3β shows similar depression-like behavioral effects as overexpression of GSK3β by Wilkinson et al., 2011 perhaps because of vast differences in stress exposure.

Previous evidence has suggested a role for GSK3β in addictive behaviors, particularly cocaine behaviors such as involvement in conditioned place preference, cocaine reward memory, cocaine hyperactivity, and cocaine behavioral sensitization (Miller et al., 2009; Xu et al., 2009; Miller et al., 2010; Miller et al., 2014; Shi et al., 2014). The current study assessed operant self-administration rather than locomotor

activity in response to stimulants or place preference behavior and is the first to directly implicate GSK3β function in the NAcSh as a mediator of cocaine self-administration. We found that GSK3β knockdown in the NAcSh of rats caused an increase in cocaine self-administration, especially at high unit doses of cocaine.

These results show that GSK3β in the NAcSh modulates behaviors related to addiction, depression, and anxiety. Further, loss of GSK3β specifically decreases spontaneous neuronal activity in TANs of the NAcSh, a neuronal population that plays an important role in these behaviors. Future understanding of the role of GSK3β signaling in the NAcSh could be informative for the development of novel pharmacotherapeutics. These results also serve to validate the first discovery-based bioinformatic strategy employed in this dissertation, a convergent transcriptomic/proteomic analysis of environmental enrichment, which identified GSK3β as a promising target.

5. STRATEGY 2: CONVERGENT FUNCTIONAL TRANSCRIPTOMICS OF ENVIRONMENTAL ENRICHMENT, COCAINE, AND GENES ENHANCED IN THE NUCLEUS ACCUMBENS SHELL

The previous chapter validated the target GSK3β, which was identified with Strategy 1 used in this dissertation. Strategy 1 investigated the cellular mechanisms of environmental enrichment at the mRNA and the protein levels from the same animals. However, the core transcriptomic analysis quantified many more genes than the core proteomic analysis. The second strategy employed in this dissertation therefore does not use the proteomic data. Rather, this chapter utilizes Strategy 2, which is a convergent functional transcriptomic analysis to identify novel targets for comorbid cocaine use disorder and mood disorders. Strategy 2 investigates the convergence of three transcriptomic datasets: mRNAs regulated by environmental enrichment, mRNAs regulated by cocaine, and a list of genes identified via a topographic transcriptomic analysis of the nucleus accumbens shell.

Evolution influences gene expression and ensures that genes are expressed largely only where they are needed. Genes with enhanced expression in the nucleus accumbens shell may be critical for reward-related behaviors and may influence the development of mood disorders and/or substance use disorders. Genes, which have proven roles in the regulation of reward related behaviors, show enhanced expression in the shell compared to other striatal regions, including cocaine and amphetamine regulated transcript (CART), serotonin receptor 2C (HTR2C), and activating transcription factor 3 (ATF3) (Douglass et al., 1995; Douglass and Daoud, 1996; McMahon and Cunningham, 1999; Green et al., 2008). We hypothesized that a prospective discovery-based analysis of the nucleus

accumbens shell will identify other genes with enhanced NAcSh expression. We utilized the Allen Mouse Brain Atlas, a freely available online resource with genome-wide *in situ* hybridization data of the entire mouse brain (mouse.brain-map.org), to probe for genes with specific expression in the nucleus accumbens shell over nearby regions, particularly the rest of the striatum.

We previously conducted a topographic transcriptomic analysis utilizing the Anatomic Gene Expression Atlas (AGEA) function of the Allen Mouse Brain Atlas, which resulted in a list of 178 genes with enhanced expression in the NAcSh (Zhang et al., 2016a). The atlas contains *in situ* hybridization experiments of the more than 20,000 genes in the adult mouse brain in 100μm coronal and 200μm sagittal slices. The AGEA is constrained only to genes that have data in the coronal plane, which only constitutes 4,376 genes; therefore, the previous analysis was restricted to this small number of genes. We aimed to expand the topographic analysis of the nucleus accumbens shell to all of the genes in the mouse genome by utilizing the correlation tool of the atlas. We conducted a topographic transcriptomic analysis of the nucleus accumbens shell with *in situ* hybridization data from the Allen Mouse Brain atlas by searching for genes with similar expression patterns as five seed genes.

We selected five genes with highly specific expression in the NAcSh with limited expression in neighboring regions, including the Islands of Calleja, nucleus accumbens core, and dorsal striatum, and used these five genes as seeds in the correlation tool of the Allen Mouse Brain Atlas. The five seed genes were selected from among the top 50 genes from the previous analysis with the AGEA. The correlation search tool compares the expression pattern of a specific gene of interest to the rest of the genome and ranks each gene by the level of correlation within a specified region (e.g. the striatum). In order to evaluate whether the NAcSh topographic gene list resulted in genes with shared functions or related pathways, we analyzed the pathway level relationships of the resulting genes with Ingenuity Pathway Analysis.

CONVERGENT FUNCTIONAL TRANSCRIPTOMICS STRATEGY

We are not only interested in identifying genes with enhanced expression in the nucleus accumbens shell, but also identification of novel targets contributing to the function of the nucleus accumbens shell. In order to narrow the promising results to one specific pathway for further study, we conducted a convergent transcriptomic analysis at the gene set level of the NAcSh topographic transcriptomics and transcripts regulated by environmental enrichment or cocaine (Zhang et al., 2016a). We used Ingenuity Pathway Analysis to investigate the pathways shared between the resulting list of genes with enhanced NAcSh expression with the enrichment and cocaine mRNA data. Environmental enrichment is an animal model resulting in protective depression and addiction phenotypes. Therefore, we hypothesized that the intersection of these three transcriptomic analyses would yield a pathway important for reward related behaviors and identify much needed novel pharmacotherapeutic targets for disorders of the reward circuitry.

METHODOLOGY: TOPOGRAPHIC TRANSCRIPTOMICS OF THE NUCLEUS ACCUMBENS SHELL

We examined the expression patterns of the top 50 genes from the previous topographic analysis of the NAcSh (Zhang et al., 2016a) and identified five genes with highly specific expression in the NAcSh with limited expression in neighboring regions (e.g. Islands of Calleja, nucleus accumbens core, dorsal striatum). The five seed genes identified are CARTPT (cocaine and amphetamine regulated transcript prepropeptide, also known as CART), NTN1 (netrin 1), SMUG1 (single-strand-selective monofunctional uracil-DNA glycosylase 1), STARD5 (steroidogenic acute regulatory (StAR) related lipid transfer domain containing 5), and STRA6 (stimulated by retinoic acid 6) (Figure 5.1). For each seed gene, we used the correlation search tool in the Allen Mouse Brain Atlas to rank all the genes in the atlas by correlation with the seed gene. We

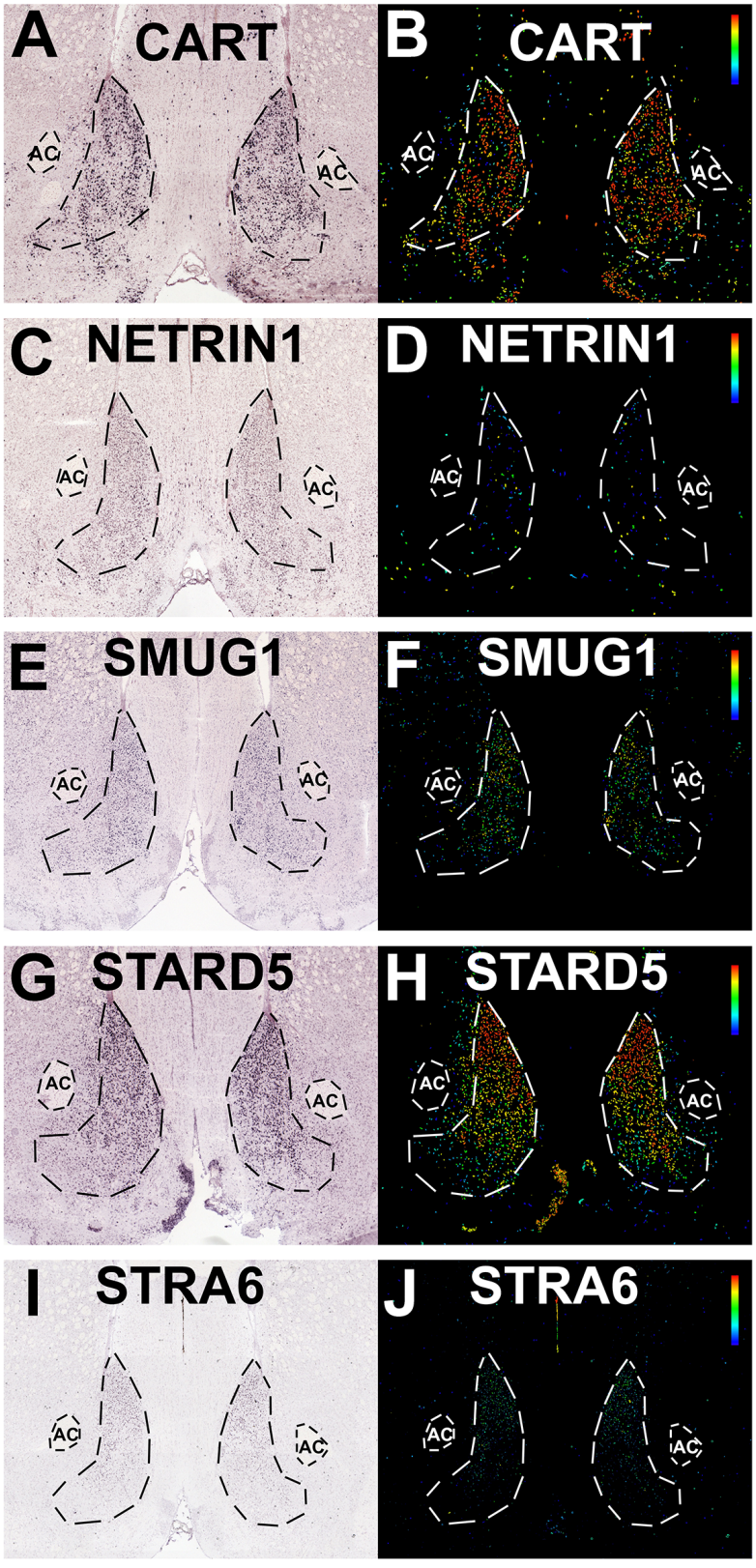

Figure 5.1 Genes with NAcSh specific expression chosen as seed genes for topographic transcriptomic analysis

In situ hybridization and expression images from the Allen Brain Atlas for cocaine and amphetamine regulated transcript (CART, Panels A-B), netrin1 (C-D), single strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1, Panels E-F), StAR related lipid transfer domain containing 5 (STARD5, Panels G-H), stimulated by retinoic acid 6 (STRA6, Panels I-J). CART: http://mouse.brain-map.org/experiment/show/72077479, NTN1: http://mouse.brain-map.org/experiment/show/74511838, SMUG1: http://mouse.brain-map.org/experiment/show/73992919, STARD5: http://mouse.brain-map.org/experiment/show/70813909, STRA6: http://mouse.brain-map.org/experiment/show/75041492

searched for each seed gene in the atlas data portal (mouse.brain-map.org), selected the coronal experiment identified from the previous analysis (CARTPT: 72077479, NTN1: 74511838, SMUG1: 73992919, STARD5: 70813909, STRA6: 75041492), and searched with the correlation tool by selecting "Striatum (STR)" in the correlation tool panel and unselecting "basic cell groups and regions". We did not select "coronal data only" in order to include all the genes in the database rather than the 4,000 with data in the coronal plane.

The correlation for each seed gene results in a rank list of all the genes in the database. We exported the top 2,000 genes from each seed gene into Excel. Data can be exported from the mouse brain atlas by selecting show 100 genes per page, viewing the data in XML format, saving the data as an XML file, and importing the XML file into Excel. Using Excel, we removed duplicate genes as well as genes that were identified in only one or two seed gene correlations. This five-pronged correlation-based search identified 1,422 mouse genes that correlated with at least three of the five seed genes. The resultant gene list was analyzed for pathway level relationships via Ingenuity Pathway Analysis.

RESULTS: TOPOGRAPHIC TRANSCRIPTOMICS ANALYSIS

Using the Allen Mouse Brain Atlas correlation tool, we identified 1,422 genes that correlated with at least three of the five seed genes (CART, NTN1, SMUG1, STARD5, and STRA6). The nucleus accumbens shell expression for each of the five seed

genes is shown in Figure 5.1. CART and STARD5 show the highest expression levels while netrin 1 and STRA6 show the lowest expression levels. We used IPA to investigate the functional relationships of the NAcSh specific genes, including overrepresented canonical pathways and upstream regulators. IPA could not identify some of the genes and were therefore excluded from the analysis. IPA therefore analyzed the pathway level relationships of 1,217 NAcSh specific genes.

Many canonical pathways were identified as significantly overrepresented in the NAcSh specific genes including *Axonal Guidance Signaling* (–log (*p*) = 3.31), *GABA Receptor Signaling* (–log (*p*) = 1.47), and $G\alpha_i$ *Signaling* (–log (*p*) = 1.42, Figure 5.2B). The nucleus accumbens utilizes GABA signaling and GPCRs as well as genes related to axonal guidance. Thus, although these are not novel canonical pathways, they bolster confidence in this set of genes. An upstream regulator that was identified is CREB1 (–log (*p*) = 6.20), Figure 5.2C), which has previously been shown to be important for the function of the nucleus accumbens (Pliakas et al., 2001; Barrot et al., 2002; McClung and Nestler, 2003; Dong et al., 2006; Green et al., 2006; Wallace et al., 2009; Green et al., 2010; Larson et al., 2011; Bilbao et al., 2014). Interestingly, a list of separate CREB target genes identified in the rat striatum with chromatin immunoprecipitation followed by sequencing (ChIP-seq, unpublished data), was also significantly overrepresented in the NAcSh specific gene set (–log (*p*) = 13.62), indicating a substantial link between topographically identified genes and CREB.

One novel canonical pathway identified in the topographic analysis, which has not been studied in neuropsychiatric disorders, is *RAR Activation* or retinoic acid receptor activation (–log (*p*) = 1.4, Figure 5.2B). Additionally, 111 of the NAcSh specific genes are downstream of retinoic acid (–log (*p*) = 2.23, Figure 5.2D). Retinoic acid receptor beta (RARB) is involved in transcriptional regulation of a multitude of target genes through interactions with retinoic acid (Niewiadomska-Cimicka et al., 2016). The current topographic transcriptomic analysis of the NAcSh also identified an overrepresentation of

transcripts in a set of RARB target genes identified via ChIP-seq from the striatum of Sprague Dawley rats (unpublished data, –log (p) = 9.45). Therefore, retinoic acid

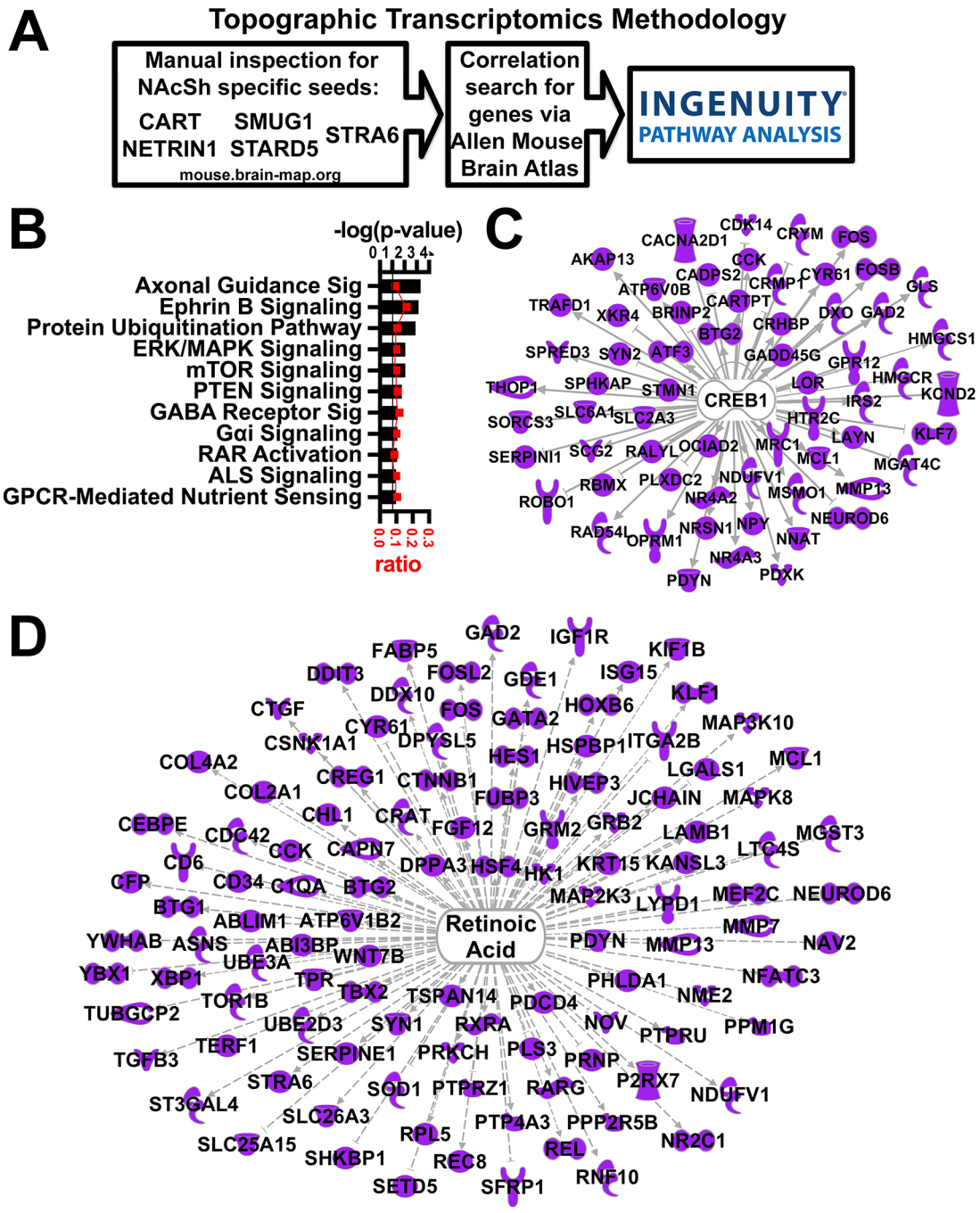

Figure 5.2 Topographic transcriptomic analysis of the nucleus accumbens shell

A. Overview of topographic transcriptomics methodology. B. A selection of significant Canonical Pathways. Horizontal axis (bars) represents –log (p-value) and ratio (red squares) of NAcSh genes to all genes in the pathway. Black line represents threshold p = 0.05. Upstream regulators identified include CREB (C) and retinoic acid (tretinoin) (D).

signaling is a promising pathway for further investigation. However, the topographic transcriptomics identified 130 canonical pathways and nearly 800 upstream regulators, not retinoic acid signaling alone. Therefore, in order to narrow the possible target pathways, we conducted a convergent functional transcriptomic analysis.

An additional goal of the current topographic analysis of the NAcSh, besides novel target identification, is to improve upon the previous topographic transcriptomic analysis, which utilized AGEA from the Allen Mouse Brain Atlas (Zhang et al., 2016a). The previous analysis was restricted to genes with *in situ* hybridization in the coronal plane, which is a fraction of the total genes in the atlas (4,376 versus >20,000). The original analysis identified 178 genes with enhance NAcSh expression whereas the new analysis identified 1,422 genes. A comparison of the canonical pathways between the original analysis and the current analysis in terms of –log (*p*) is shown in Figure 5.3.

In addition to identifying more genes, the new analysis also identified more significantly overrepresented canonical pathways. The original analysis identified 14 pathways not identified in the new analysis, while the new analysis identified 116 unique pathways. Fourteen canonical pathways were identified in both analyses.

Interestingly, both analyses led to the identification of retinoic acid signaling as having genes with enhanced expression in the NAcSh (Zhang et al., 2016a). The core retinoic acid pathway was significant in both analyses (original analysis –log (*p*) = 4.78, new analysis –log (*p*) = 1.86). However, the previous analysis did not find a significant overrepresentation of the NAcSh enhanced genes in the *RAR Activation* pathway (–log (*p*) = 1.23) whereas *RAR Activation* was significant in the new analysis (–log (*p*) = 1.4). Both analyses also identified STRA6 and FABP5 as NAcSh enhanced genes, however, only the new analysis identified ALDH1B1, RAR gamma, and RXRα, while only the original analysis identified RARB and CYP26B1. In the new analysis, retinoic acid receptor beta (RARB) correlated with only one seed gene. Upon inspection of RARB in the Allen Brain Atlas, RARB is expressed in the dorsal striatum at similar levels to the

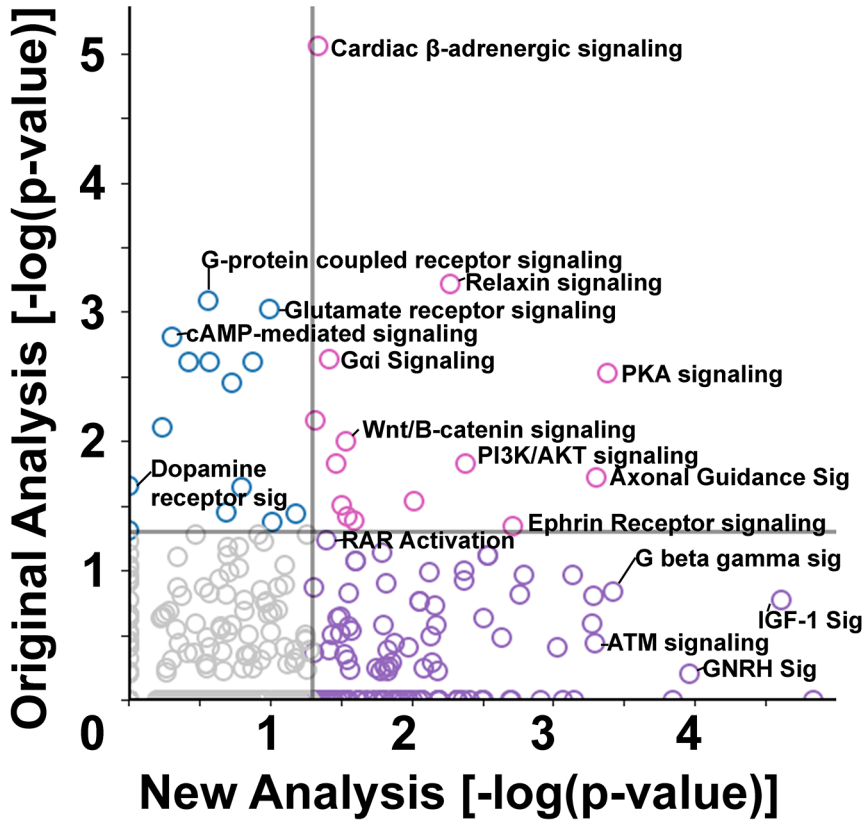

Figure 5.3 Comparison of canonical pathways between previous topographic analysis and current topographic analysis

Reference lines indicate significant –log(p-value). Blue symbols indicate canonical pathways that were identified as significant in the original analysis and not the new analysis. Pink symbols indicate significant canonical pathways in both analyses. Purple symbols indicate canonical pathways that were significant only in the current analysis. Grey symbols were not significant in either analysis.

shell. CYP26B1 (cytochrome p 450 family 26 subfamily b member 1) is involved in the degradation of retinoic acid, and knockdown of CYP26B1 in the NAcSh (i.e. increased retinoic acid) causes increases in cocaine self-administration (Zhang et al., 2016a). The *in situ* hybridization data for CYP26B1 shows that CYP26B1 is expressed at a very low level in the NAcSh, which may have contributed to it not being identified in the new analysis.

METHODOLOGY: CONVERGENT FUNCTIONAL TRANSCRIPTOMIC ANALYSIS

In order to identify the most promising pathway for further study, we conducted a convergent functional transcriptomic analysis at the gene set level by comparing the canonical pathways and upstream regulators identified in the topographic transcriptomic analysis of the NAcSh as well as the transcriptomic analysis of environmental enrichment and cocaine with Ingenuity Pathway Analysis (Zhang et al., 2016a). The environmental enrichment versus isolation analysis includes 2,940 significantly regulated transcripts while the cocaine versus saline analysis includes 1,138 transcripts. As mentioned above, 1,217 NAcSh specific genes were analyzed with IPA.

A standard approach to identify a target would be to examine the three transcriptomic analyses at the individual gene level. A target would be chosen from the 57 individual genes that are identified in all three transcriptomic analyses (Figure 5.4). However, anxiety, depression, and substance use disorders are not monogenetic disorders and involve alterations in a multitude of genes and signaling pathways. Therefore, we conducted a convergent transcriptomic analysis at the pathway level.

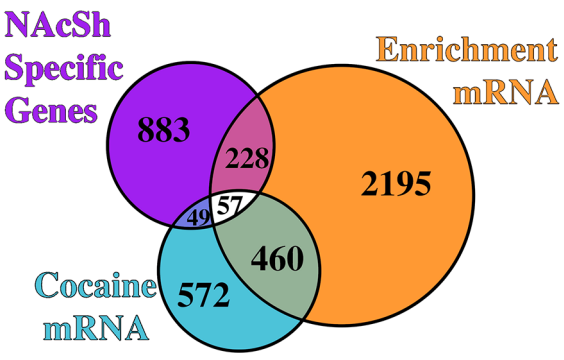

Figure 5.4 Convergent transcriptomics at the individual gene level

RESULTS: CONVERGENT FUNCTIONAL TRANSCRIPTOMICS OF ENVIRONMENTAL ENRICHMENT, COCAINE AND NACSH SPECIFIC GENES

As shown in Figure 5.5A, we found significant overlap of canonical pathways via this convergent approach. Sixty-eight canonical pathways were identified in all three transcriptomic analyses (Figure 5.5A).

One pathway identified in all three transcriptomic analyses is *RAR Activation* (EC −log (p) = 6.9), Cocaine −log (p) = 1.92), NAcSh specific −log (p) = 1.4)). The core retinoic acid signaling pathway is shown in Figure 5.5 B-D, overlaid with NAcSh specific genes identified with the topographic transcriptomic analysis, enrichment mRNA, or cocaine mRNA data from the previous transcriptomic analysis (Zhang et al., 2016a). Briefly, the core retinoic acid pathway illustrates the various genes involved in the trafficking and signaling of retinoic acid. Retinoic acid (RA) enters the cell or is synthesized from retinol in the cytoplasm. RA can be degradation into retinoic acid metabolites by CYP26B1, or associate with binding partners FABP5 (fatty acid binding protein 5) or CRABP2 (cellular retinoic acid binding protein 2). FABP5 delivers RA to peroxisome proliferator-activated receptor delta (PPARD) and retinoid x receptors (RXR), where it regulates transcription of genes with PPAR response elements (PPREs) (Schug et al., 2007). In contrast, CRABP2 delivers RA to RXR and retinoic acid receptors (RAR), regulating transcription through retinoic acid receptor response elements (Schug et al., 2007). In this manner, retinoic acid regulates the expression of hundreds of downstream targets.

As shown in Figure 5.5B, NAcSh specific genes are involved in the trafficking of retinol and RA (STRA6 and FABP5) as well as involved in RA transcriptional regulation (RAR and RXR). Transcripts related to the synthesis of retinoic acid are upregulated by environmental enrichment (RBP4 (retinal binding protein), RBP1, and RDH (retinol dehydrogenase), Figure 5.5C). The RA binding partners, FABP5 and CRABP2 are also

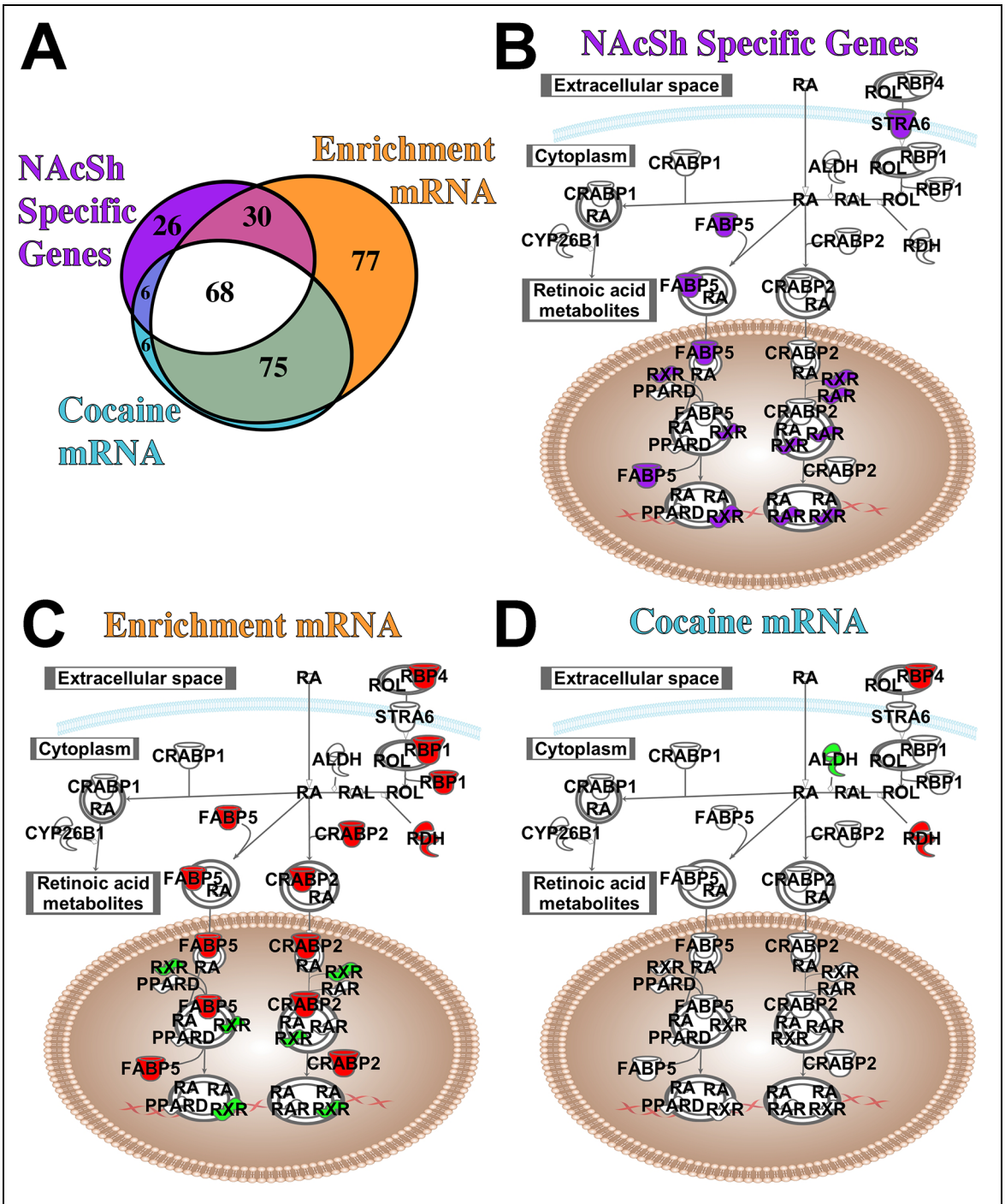

Figure 5.5 Convergent functional transcriptomics of enrichment mRNA, cocaine mRNA, and NAcSh specific genes at gene set level reveals retinoic acid signaling pathway

A. Venn diagram of overlapping canonical pathways in three transcriptomics gene sets. Retinoic acid core signaling pathway overlaid with NAcSh specific genes (B), enrichment mRNA (C), or cocaine mRNA (D). Purple symbol indicates NAcSh enhanced expression. Green symbol indicates downregulation by enrichment or cocaine while red symbol indicates upregulation.

upregulated by enrichment while RXR is downregulated (Figure 5.5C, (Zhang et al., 2016a). Retinol binding protein 4 (RBP4) is upregulated by cocaine (Figure 5.5D). Retinol dehydrogenase (RDH), which oxidizes retinol into retinaldehyde, is also upregulated by cocaine while retinaldehyde dehydrogenase (ALDH), which converts retinaldehyde to retinoic acid, is downregulated (Figure 5.5D).

Additionally, all three transcriptomic analyses identified many downstream retinoic acid target genes as significantly overrepresented. Cocaine identified 118 significantly regulated retinoic acid targets genes (–log (p) = 5.80), enrichment regulated 234 downstream genes (–log (p) = 2.44), and 111 NAcSh specific genes are downstream of retinoic acid (–log (p) = 2.23). Finally, enrichment and cocaine also regulated a significant portion of the RARB ChIP-seq target genes (EC –log (p) = 39.3, Cocaine –log (p) = 1.80), just as RARB target genes were significantly overrepresented in the NAcSh specific gene list (–log (p) = 9.45).

TARGET VALIDATION OF RETINOIC ACID SIGNALING: FATTY ACID BINDING PROTEIN 5

Retinoic acid is a metabolite of retinol, or vitamin A, and is critically important for a variety of functions, especially embryonic pattern development, development of the nervous system, as well as maintaining the adult nervous system (Maden, 2007; Duester, 2008). Retinol and retinoic acid are extracted from food and released by the liver into the bloodstream. Retinoic acid is found throughout the brain, including the striatum (Kane et al., 2008). Retinoic acid exerts effects by binding to ligand activated nuclear receptors (including RARs, RXRs, and PPARs) and typically activates transcription of hundreds of downstream targets (Balmer and Blomhoff, 2002; Maden, 2007). Downstream targets of retinoic acid are involved in a variety of functions and several are related to reward signaling, such as dopaminergic receptors and cholinergic receptors (Balmer and Blomhoff, 2002; Niewiadomska-Cimicka et al., 2016).

Strategy 2, the convergent transcriptomic strategy, clearly identified retinoic acid signaling as a promising pathway for further investigation. Several genes within the core pathway were regulated by environmental enrichment or cocaine or were identified in the topographic transcriptomics of the NAcSh. Even though Strategy 2 clearly identified retinoic acid signaling, few individual transcripts overlapped in all three of the transcriptomic datasets. Therefore, we chose to manipulate one target in the core retinoic acid signaling pathway that was regulated by environmental enrichment, shows highly shell specific expression, and is also a critical retinoic acid binding partner. The target is fatty acid binding protein 5 (FABP5). The *in situ* hybridization and expression images for FABP5 from the Allen Mouse Brain Atlas are shown in Figure 5.7 Panels A and B. FABP5 binds retinoic acid and delivers it to peroxisome proliferator-activated receptor delta (PPARD, also known as PPAR beta) and causes transcription of genes with peroxisome proliferator activator response elements rather than retinoic acid response elements (RARE) (Schug et al., 2007). In order to validate this novel pathway, we knocked down FABP5 in the NAcSh *in vivo* via RNA interference and examined cocaine taking and seeking behaviors.

Animals

For knockdown of FABP5 and behavioral tests, male Sprague-Dawley rats were obtained at 225-249g. Rats were pair-housed and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved colony and procedures were approved by the UTMB Institutional Animal Care and Use Committee and conform to the NIH Guide for the Care and Use of Laboratory Animals.

Knockdown of FAPB5 in the rat NAcSh

In order to decrease the expression of FABP5 in the NAcSh, 5 shRNA sequences were designed to target the rat FABP5 coding sequence and then constructed. The 5

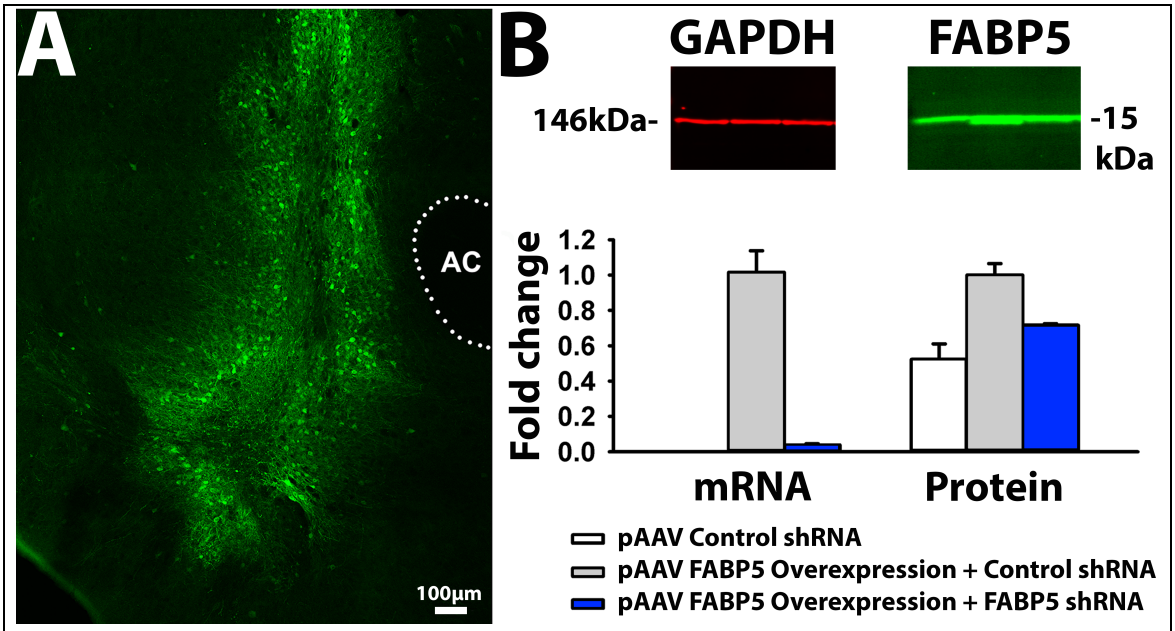

Figure 5.6 Validation of novel AAV shFABP5 vector

A. Representative immunofluorescence (eGFP) of viral vector in the NAcSh (AC=anterior commissure). B. In vitro validation fold change of FABP5 mRNA (mean ±SEM) and protein (mean ±SEM) in HEK293 cells transfected with pAAV-control, pAAV-control with FABP5 overexpression plasmid, or pAAV-shFABP5 and FABP5 overexpression plasmid normalized to GAPDH. Inset shows Western blot for FABP5 and GAPDH.

constructs were co-transfected into HEK293 cells with a plasmid overexpressing rat FAPB5 to determine the most efficient hairpin construct (Figure 5.6). The most efficient hairpin (5'.GGAAGGGAAAGAAAGCACGATAAC.3'; 96% knockdown of mRNA and 28% knockdown of protein, Figure 5.6B) was packaged into an AAV2 capsid (UNC Vector Core) that co-expresses eGFP. The viral titer was determined by the UNC Vector core via qPCR (control shRNA titer 1.4 x 10^{12}vg/ml; shFABP5 titer 4.6 x 10^{12}vg/ml). The shFABP5 vector or control non-targeted hairpin vector (n=10-12 each) was injected bilaterally into the rat NAcSh with methods described previously (Zhang et al., 2016a; Crofton et al., 2017b). Control and FABP5 shRNA vectors were injected bilaterally using coordinates AP=1.7, L=2.2, D=-6.7 at a 10° lateral angle (1µl/side over 10 min) under ketamine/xylazine anesthesia. The injection needles remained in place for 10 minutes post-injection to allow for spread of the vector throughout the NAcSh. Three weeks after

surgery rats underwent a number of depression, anxiety, and motivation behavioral tasks, including locomotor activity, elevated plus maze, sucrose neophobia (1% liquid), sucrose preference (1% sucrose over water), social contact, cold-stress induced defecation, forced swim test, operant sucrose pellet responding at 85% free-feed body weight and again at 100% free-feed body weight. None of these tests produced statistically significant results (Zhang et al., manuscript in review, data not shown).

Cocaine self-administration

At 6 weeks post vector injection, rats were implanted with a Silastic catheter (0.2 mm I.D.) into the jugular vein, exiting the skin via an external port on the rat's back. Catheters were infused with 0.1 mL of flushing solution (heparin 30.0 U/mL, ticarcillin 250,000 U/mL, streptokinase 8,000 IU/mL, dissolved in sterile saline) daily in order to prevent infection and promote catheter patency for the duration of the study. Rats recovered for one week prior to acquisition of cocaine self-administration. Accurate placements of the viral vectors were verified immunohistochemically after the conclusion of behavioral testing.

ACQUISITION: One week after catheter surgery, all rats were placed in operant chambers (30 x 24 x 21 cm; Med-Associates, St. Albans, VT) and allowed to self-administer 0.2 mg/kg/infusion unit dose of cocaine for 2 hours per session for 5 days; then 0.5 mg/kg/infusion for 3 days on an fixed ratio (FR1) schedule. Each infusion was delivered intravenously in a volume of 0.1 ml over 5.8 sec. The infusion was signaled by illumination of two cue lights for 20 sec, which signaled a timeout period during which no further infusions could be obtained.

FIXED RATIO DOSE RESPONSE: Each rat (control shRNA or FABP5 knockdown) was allowed to self-administer 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.0075, 0.00325 mg/kg/infusion cocaine within-session in descending order on an FR1 schedule each day for five consecutive days. Rats self-administered each dose of cocaine for 30 min.

EXTINCTION: Stably responding rats underwent a within-session extinction procedure for 3 days. All rats were allowed to self-administer 0.5mg/kg/infusion unit dose of cocaine under an FR1 schedule for 1 hour followed by extinction for 3 hours. During the extinction period, lever presses resulted in cue-light illumination under an FR1 schedule, but the pump did not deliver cocaine infusions.

REINSTATEMENT: All rats received 0.5mg/kg/infusion unit dose of cocaine under an FR1 schedule for 1 hour followed by 3 h of extinction. Next, all rats received an IP injection of cocaine of one of five doses (0, 2.5, 5, 10, 20mg/kg) in a random order for each rat across the five sessions, followed by 3 h of reinstatement responding.

Statistical Analysis of Behavior

Two-factor analyses of variance (ANOVAs) and two-factor repeated measures ANOVAs were performed to compare four treatment groups. Significance between only two conditions was analyzed using a Student's t-test. All t-test data passed the Shapiro-Wilk test of normality. All data are expressed as mean ±SEM. Statistical significance was set at $p<0.05$.

Results: Knockdown of fatty acid binding protein 5

The knockdown efficiency of the novel shFABP5 vector was validated in HEK293 cells and showed more than 96% knockdown at the mRNA level and approximately 60% knockdown for protein (Figure 5.6B). Nine weeks after viral vector injection of shFABP5 or control vector, all rats started cocaine self-administration (Figure 5.7C). For acquisition of cocaine, there were significant main effects of Session ($F(4, 80) = 8.031$, $p < 0.001$) and Vector ($F(1, 20) = 7.238$, $p = 0.014$) at the low unit dose of cocaine (0.2mg/kg/infusion, Figure 5.7D). There was also a significant main effect of Session ($F(2, 40) = 6.230$, $p = 0.004$), with a trend for decreased maintenance responding in FABP5 knockdown rats ($F(1, 20) = 4.176$, $p = 0.054$) at a high unit dose

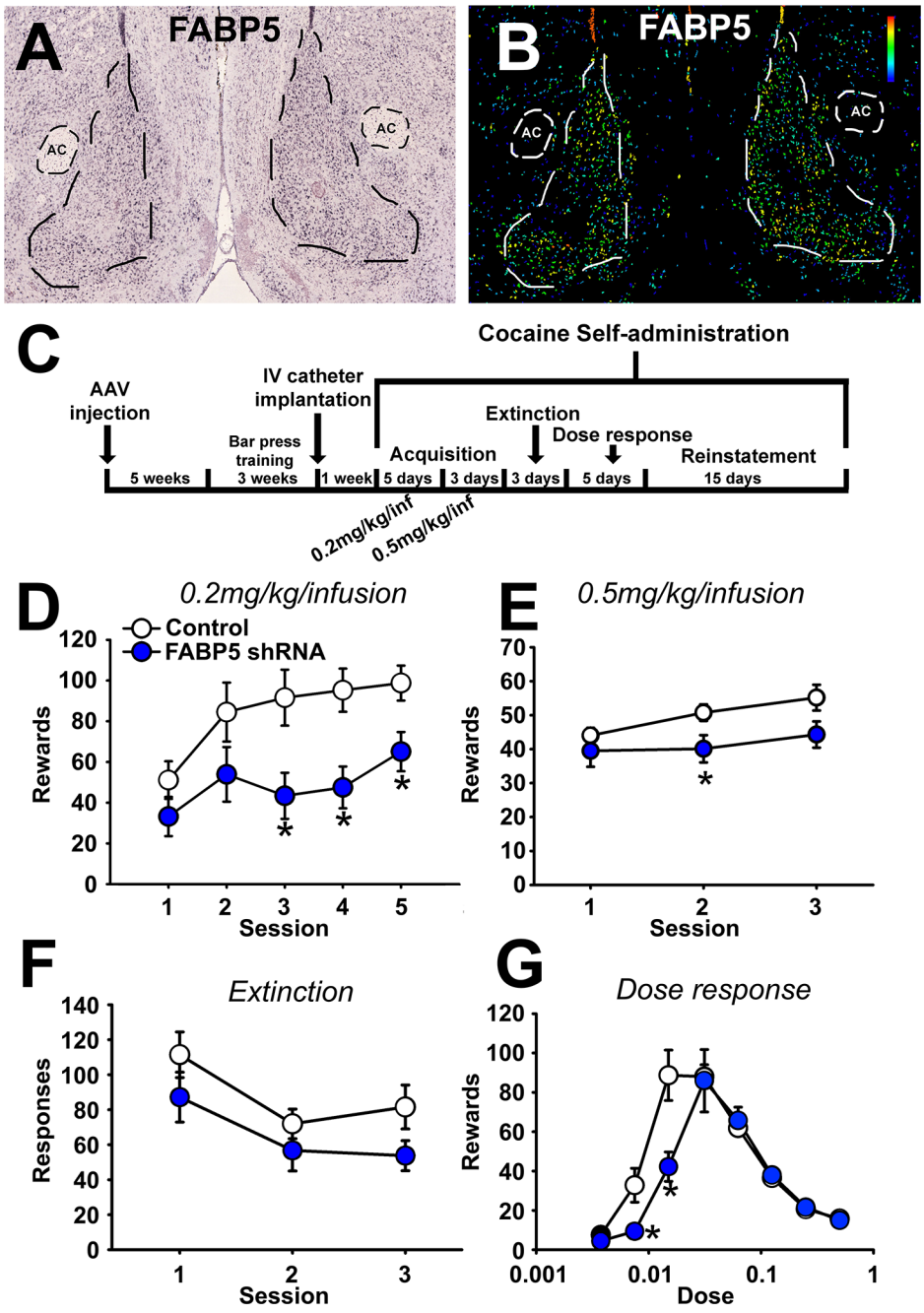

Figure 5.7 Fatty acid binding protein 5 was identified as a NAcSh specific gene and knockdown of FABP5 mimics the resilient addiction phenotype of environmental enrichment

In situ hybridization (A) and expression (B) images from the Allen Brain Atlas for fatty acid binding protein 5 (FABP5) http://mouse.brain-map.org/experiment/show/70634396. (C) Diagram of cocaine self-administration methods. Acquisition at 0.2mg/kg/infusion (D) and 0.5mg/kg/infusion (E), extinction (F), and dose response (G) of cocaine self-administration in control injected or FABP5 knockdown rats.

of cocaine (0.5mg/kg/infusion, Figure 5.7E). As a model of craving, the cocaine within-session extinction paradigm revealed that knocking down FABP5 in the NAcSh produced a trend for decreased drug-seeking behavior ($F(1, 21) = 3.083$, $p = 0.094$; Figure 5.7F). Knocking down FABP5 in the NAc shell caused a rightward shift of the ascending limb of the dose response curve with a significant interaction of Dose X Vector ($F(7, 119) = 4.713$, $p < 0.001$; Figure 5.7G), indicating decreased responding to low unit doses of cocaine. Knocking down *Fabp5* in the NAc shell did not change the responding to cocaine-induced reinstatement (data not shown). Overall, rats with FABP5 knocked down in the NAcSh exhibited decreased cocaine taking that was more pronounced at lower unit doses and possibly decreased cocaine seeking behavior (i.e. a trend).

DISCUSSION

We conducted a convergent transcriptomic analysis investigating the intersection of environmental enrichment, cocaine, and a topographic transcriptomic analysis of the nucleus accumbens shell. The convergent transcriptomic analysis was conducted in order to identify novel pathways with targets for therapeutic development for substance use disorders, cocaine use disorder in particular. This approach clearly identified retinoic acid signaling. Therefore, we knocked down expression of the retinoic acid binding partner FABP5 in the nucleus accumbens shell of rats via a novel adeno-associated viral vector. FABP5 showed selective expression in the nucleus accumbens shell, was regulated by environmental enrichment, and is an important retinoic acid binding partner. However, FABP5 was not regulated by cocaine, highlighting the utility of a pathway level investigation. A traditional convergent approach, investigating the overlap at the individual gene level of these three transcriptomic datasets, would not have led to the selection of FABP5. Rats with FABP5 knockdown in the NAcSh showed decreased cocaine taking behavior as compared to control rats, mimicking the resilient addiction phenotype of environmentally enriched rats (Green et al., 2010). Thus, retinoic acid

signaling was confirmed as an important pathway in the nucleus accumbens shell for the regulation of cocaine-seeking behaviors.

The current topographic analysis of the nucleus accumbens shell determined a list of genes that correlated with at least three highly NAcSh specific genes (CART, NTN1, SMUG1, STARD5, and STRA6). We identified the five seed genes by examining the top 50 genes from a previous topographic analysis of the NAcSh (Zhang et al., 2016a). This original analysis was restricted to genes in the Allen Mouse Brain Atlas with coronal experiments, which is only a fraction of the genes in the atlas (4,376 versus >20,000). The goal of the current topographic NAcSh analysis was to improve upon this initial analysis.

The current analysis is a genome-wide approach with the correlation tool while the original analysis was a much less comprehensive approach with AGEA. AGEA searches for genes with correlated expression in a specific seed coordinate versus the rest of the brain (Lein et al., 2007; Ng et al., 2009). The entire brain is delineated into 200μm^3 voxels and can be searched with any individual voxel. However, AGEA relies on the accuracy and uniformity of all the brain slices as well as precise alignment of each voxel between animals. Additionally, results with AGEA depend greatly on the specific voxel selected. In the original analysis, extensive manual culling of the data was required to reduce the number of false positives as a result of artifacts, such as bubbles, or experiments that were skewed by high expression in a neighboring region (e.g. NAc core, diagonal band nucleus, septal nucleus, olfactory tubercles, Islands of Calleja). The current analysis with the correlation tool does not rely on an individual voxel but rather searches for genes with similar expression patterns in the nucleus accumbens compared with the seed gene.

The current topographic analysis is therefore an improvement over the original analysis as the current analysis is more subjective and includes all the genes in the mouse genome. The current analysis resulted in identification of many more genes as well as

many more canonical pathways. One of the canonical pathways identified only in the new analysis is *GNRH signaling* or gonadotropin-releasing hormone signaling. *GNRH signaling* has previously been suggested to be involved in both depression and addiction by previous bioinformatic analyses (Li et al., 2008; Gormanns et al., 2011), and is a promising target for future investigation. Even though the original analysis identified pathways that were not identified by the new analysis, some are redundant with pathways identified in the new analysis. For example, *G-protein coupled receptor signaling* was identified in the original analysis only, but both analyses identified *Gαi signaling,* while only the new analysis identified the specific G-protein coupled receptor pathways *Gαq signaling, Gα12/13 signaling,* and *G beta gamma signaling*. The new analysis identified multiple specific g-protein coupled receptor pathways rather than the general pathway identified in the original analysis, illustrating the utility of the new analysis. Additionally, half of the pathways identified by the original analysis were also identified by the new analysis (Figure 5.3). Therefore the current analysis indicates that using the correlation based approach is more effective than using the current restricted AGEA.

This unbiased topographic transcriptomic analysis would not be possible without the genome-wide database of *in situ* hybridization data from the Allen Brain Institute. However, a caveat of this chapter is that the atlas of brain tissue is from mice while the FABP5 knockdown experiment was conducted in rats, as was the transcriptomics of enrichment and cocaine. Despite the differences in species, we were able to identify a novel pathway with Strategy 2 and when manipulated in rats, caused a decrease in cocaine taking behaviors. This provides confidence that retinoic acid signaling will prove important for drug taking behaviors in humans.

Although FABP5 knockdown in the NAcSh altered cocaine taking, knockdown rats did not show alterations in anxiety-like or depression-like behaviors. Perhaps FABP5 needed to be knocked down for an extended period to alter behavior and thus showed alterations only in the final behavior investigated (cocaine self-administration, 9 weeks

after vector injections) and not anxiety- and depression-like behaviors. This would indicate possible involvement of other types of regulation such as epigenetic modifications. However, behavioral experiments commenced only after the vector reached peak expression levels (>2 weeks).

A more likely explanation for a lack of effect of FABP5 knockdown on anxiety- and depression-like behaviors is that the other retinoic acid binding partner, CRABP2, or some other mechanism, compensated for the loss of FABP5. RA signaling does have demonstrated downstream effects on transcription through PPARD (via FABP5) but also through RARs (via CRABP2) (Schug et al., 2007). Perhaps RA signaling via CRABP2/RARs compensated for the loss of FABP5 in the anxiety- and depression-like tests but not cocaine-taking. Previous evidence suggests that RA signaling, through CRAPB2/RAR, controls local protein translation in dendrites by associating in RNA granules in dendrites (Maghsoodi et al., 2008; Chen et al., 2014). This non-transcription related or non-genomic effect of RA signaling through RARs was previously shown to regulate synaptic strength by increasing the expression of GluA1-containing AMPA receptors, which lack the GluA2 subunit and are permeable to calcium (Aoto et al., 2008; Chen et al., 2014). RA signaling through this non-genomic effect may have compensated for the loss of FABP5. However, the non-genomic effects of FABP5 have not been examined. Future studies should examine the role of RA signaling on neuronal firing and synaptic plasticity in the NAcSh as well as investigating the genomic and non-genomic effects of CRABP2/RAR and FABP5/PPARD. A better understanding of the role of RA signaling on anxiety-, depression-, and addiction-like behaviors is necessary to develop effective pharmacotherapeutics targeting RA signaling for anxiety, depression, and cocaine use disorder.

Although we did not see effects on anxiety- and depression-like behaviors, the current knockdown of FABP5 in the NAcSh does indicate that inhibition of FABP5 is a promising therapeutic target for cocaine use disorder. Knockdown of FABP5 in the

NAcSh caused a rightward shift in the ascending limb of the dose response function, indicating an alteration in the threshold of reinforcement for cocaine. Small molecules targeting FABP5 may increase the threshold of reinforcement of cocaine in humans as well. This strategy could be a potent therapeutic for treatment-seeking individuals with cocaine use disorder to aid in maintaining abstinence, even in the face of relapse to cocaine use. The effect of specific small molecules inhibiting fatty acid binding proteins on cocaine self-administration should be assessed, especially cocaine-induced reinstatement responding after a long period of abstinence. A promising lead molecule for further study is the fatty acid binding protein inhibitor SBFI26. SBFI26 inhibits FABP5 and FABP7 in the brain and has been shown to have analgesic properties but minimal abuse potential (Kaczocha et al., 2014; Thanos et al., 2016). The effect of SBFI26 on cocaine taking behavior and cocaine-induced reinstatement after prolonged abstinence should therefore be assessed.

An important consideration for FABP5 as a therapeutic target is assessment of global FABP5 inhibition, as FABP5 also shows expression in other brain regions, in particular, the pyramidal layer of the hippocampus, the dentate gyrus, the medial habenula, and cerebellum (Allen Mouse Brain Atlas). Inhibition of FABP5 could therefore alter learning and memory or even coordinated movement. FABP5 may also play a role outside of the central nervous system as well. Therefore, the effects of systemic inhibition of FABP5 should be examined.

The current study conducted within-session reinstatement after 1 hour of cocaine taking followed by 3 hours of extinction and did not find effects of FABP5 knockdown in this within-session reinstatement procedure. However, we did not assess effects of FABP5 knockdown on cocaine-induced reinstatement after a prolonged period of abstinence, which would more closely resemble a treatment-seeking individual with cocaine use disorder. Future studies should therefore examine the role of FABP5 on

reinstatement after several days of abstinence via shRNA targeting of FABP5 as well as small molecule inhibitors of FABP5, such as SBFI26.

The convergent transcriptomics strategy examining transcripts regulated by cocaine, environmental enrichment, and selective expression in the NAcSh indicated RA signaling as a promising treatment target for comorbid mood disorders and cocaine use disorder. Manipulation of FABP5 provides evidence that manipulation of retinoic acid signaling is a promising target for cocaine use disorder. To aid pharmacotherapeutic development for these comorbid disorders, further investigation should focus on understanding the genomic and non-genomic roles of retinoic acid signaling on cocaine taking behaviors and affective behaviors in the NAcSh as well as throughout the CNS and periphery.

6. GENERAL DISCUSSION

This dissertation examines the nucleus accumbens and an animal model of resilience to depression and addiction in order to identify and validate novel targets for comorbid substance use disorders and mood disorders. Two discovery-based strategies were employed including a convergent transcriptomic/proteomic analysis of environmental enrichment and cocaine (Strategy 1, Chapter 3) and a convergent functional transcriptomics analysis of environmental enrichment, cocaine, and the nucleus accumbens shell (Strategy 2, Chapter 5). The discovery-based strategies resulted in the hypotheses that *AKT Signaling* (Strategy 1) and *RA Signaling* (Strategy 2) have roles in the regulation of behavior in the NAcSh. These signaling pathways were validated through *in vivo* knockdown of GSK3β (Chapter 4) and FABP5 (Chapter 5) in the NAcSh through AAV-mediated RNA interference. NAcSh knockdown of GSK3β mimics the susceptible depression and addiction phenotypes seen in isolated rats while NAcSh knockdown of FABP5 mimics the resilient addiction phenotype of environmentally enriched rats. These results support the overall hypothesis that novel targets for comorbid neuropsychiatric disorders can be identified through discovery-based investigation of environmental enrichment and the nucleus accumbens. This dissertation provides rationale for further investigation of these targets as well as demonstrates the utility of these bioinformatic strategies and develops novel tools, which can both be applied broadly.

DISCOVERY-BASED STRATEGIES FOR NOVEL TARGET IDENTIFICATION

Technological and methodological advances have made high throughput quantification of RNA and protein more widely utilized and has resulted in many large-scale datasets with thousands of potentially promising targets. It is not feasible to

investigate and validate every target; therefore, novel strategies to narrow the many exciting targets are necessary. Difficulty remains in determining the biological relevance of the resulting data and identifying the most promising target for further investigation. However, the two discovery-based strategies used in this dissertation are a step forward in integrating genome-wide datasets and can be applied to a multitude of experiments to accelerate target identification.

Examining the convergence of multiple -omics datasets at the pathway level as in Strategy 2, as well as multiple levels of analysis (e.g. transcriptomics, proteomics, genomics, metabolomics) as in Strategy 1, can lead to novel hypothesis generation and thus a better understanding of the underlying neurobiology. The goal of a traditional omics analysis is to identify one specific target and ensure that is a "true positive" (i.e. it is identified as significantly regulated in the analysis and also has biological significance) by minimizing Type I error (i.e. "false positives"). Unfortunately, these datasets are very large and involve many comparisons, which greatly increases the Type I error rate. Conservative approaches aim to reduce Type I error by increasing the p-value stringency (e.g. Bonferroni correction) and/or setting a fold change threshold for exclusion of individual targets. However, a fold change cutoff and increased p-value stringency greatly increase the Type II error rate (i.e. false negatives). A fold change cutoff is especially problematic for data derived from brain tissue, because the brain is exquisitely regulated and does not often show drastic expression changes; even small alterations in the brain can have a strong effect on the function of the cell. Bioinformatics software is designed to account for the multiple comparisons inherent in omics datasets, therefore a focus on the gene set level regulation does not require additional p-value stringency. We focused on pathways that were regulated by multiple manipulations or at multiple levels of analysis, as these pathways are more likely to not only be regulated in the dataset, but also be biologically relevant. The *PI3K/AKT signaling* pathway was regulated by environmental enrichment and cocaine at both the mRNA and protein levels, and AKT

was identified as an upstream regulator by enrichment and cocaine at the mRNA level. Additionally, IPA and GSEA both identified AKT signaling. These results provide confidence that AKT signaling is important to the function of the nucleus accumbens shell, especially in regulating behavior. Similarly, retinoic acid signaling was regulated by enrichment and cocaine as well as having a significant number of genes with enhanced expression in the shell. Retinoic acid signaling also appeared promising because it was regulated in a coordinated fashion by environmental enrichment. Transcripts involved in the trafficking of retinol, the synthesis of retinoic acid, and retinoic acid binding partners were all increased by environmental enrichment (Figure 5.5C). We would expect that if a pathway were a false positive, the individual genes would be randomly distributed, whereas a true positive would be regulated in such a way as to have biological significance (i.e. coordinated regulation or consistently identified in multiple analyses).

The strategies employed are designed to compensate for inherent issues in each separate analysis by utilizing convergent approaches. Traditional omics strategies and even traditional convergent functional genomics strategies are focused on the identification of a single target that shows regulation in a specific phenotype. However, an individual gene could be critical for the underlying biology, yet not be regulated at one level or another (i.e. false negative). For example, Strategy 1 found that mRNAs and proteins from one brain region, even from the same animals, show little correspondence at the individual target level. Proteins are translated from mRNA, which is transcribed from DNA; however, mRNA and protein levels, turnover, and activity can be regulated by a variety of processes such as transcription factors, microRNAs, alternative splicing, phosphorylation, ubiquitination, etc. Although AKT signaling was regulated by enrichment and cocaine, GSK3β was only identified in the transcriptomic analysis. Through the target validation of GSK3β, we have shown that it is a true positive in the transcriptomic analysis. It is interesting to note that the strategies we employed do not differentiate between a target that is not regulated in the dataset and a target that is not

identified or quantified. Therefore, although we aimed to quantify the mRNA and protein at a genome-wide level, in actuality, we quantified 14,309 mRNAs and 1917 proteins in the transcriptomic and proteomic analyses of environmental enrichment and cocaine (Chapter 3). This highlights the utility of these bioinformatic strategies, as despite this lack of identification, we are able to identify pathways that are regulated at both the mRNA and the protein levels.

Another factor affecting the identification of an individual target at the mRNA and protein levels is the timing of the mRNA and protein extraction. We chose to harvest the tissue three hours after the beginning of the final self-administration session. Future studies would be improved by harvesting the tissue at more than one time point and comparing between different times. Although there was low correspondence at the individual target level, this approach demonstrated that mRNA and protein collected at the same time can show correspondence at the pathway or gene set level. Therefore, even though the individual targets have low correspondence, a transcriptomic/proteomic analysis can identify similar promising signaling pathways. Similar to Strategy 1, Strategy 2 identified retinoic acid signaling, but FABP5 was only identified in two out of three transcriptomic analyses. A traditional approach would not have chosen FABP5 or GSK3β for further investigation, highlighting the utility of these strategies.

An issue plaguing these bioinformatic strategies is the reliance on comparing the query datasets with databases of known interactions between genes. IPA is continually updating interactions between genes in the IPA curated knowledge base based on new published data. However, much of this information is based on interactions from cell culture, especially cancer cell lines, rather than brain tissue and neurons. Therefore, these identified interactions and associations may not hold true for brain-derived datasets. Additionally, interactions between genes that may prove critical for these complex disorders may be unidentified and therefore not included in the databases. This dissertation utilized multiple software programs that use different databases in order to

minimize these discrepancies. Future studies would be improved by a similar use of multiple databases or the development of a neuroscience specific database of gene interactions.

Applications of bioinformatic strategies employed and future studies

As the use of large-scale analyses continues to expand, the amount of data exponentially increases and more biological datasets are available in repositories. We deposited the transcriptomic data for environmental enrichment and cocaine into the NCBI Gene Expression Omnibus and the proteomic data for environmental enrichment and cocaine into the ProteomeXchange database. Many other transcriptomic and proteomic datasets are freely available in these and other databases. The strategies employed here can be applied broadly and used in the future to examine already existing freely-available datasets as well as datasets generated in the future. These types of *in silico* secondary analyses of published datasets have the additional benefit of reducing the number of animals used. However, *in silico* strategies as well as both strategies used in this dissertation only indicate promising pathways that must be validated, such as with *in vivo* knockdown using RNA interference, as used here.

This dissertation has demonstrated the utility of integrative and convergent bioinformatic approaches to identify promising pathways for further analysis as well as understanding genome-wide effects of manipulations. We showed that Strategy 1 can be used to examine a manipulation at multiple levels of analysis (mRNA and protein) while Strategy 2 can be used to identify targets with high confidence through multiple lines of evidence. Strategy 1 and Strategy 2 employed here can therefore be used in the future to examine substance use disorders and mood disorders, as well as applied to the study of other animal models of neuropsychiatric disorders.

TRANSLATION OF BIOINFORMATIC STRATEGIES FOR SUBSTANCE USE DISORDERS

For example, both strategies could be applied to examine substance use disorders in other ways. Strategy 1 could be utilized to compare the epigenetic and transcriptomic effects of cocaine as several datasets in the Gene Expression Omnibus database examine DNA methylation changes in the nucleus accumbens after cocaine exposure. Strategy 2 could be used to compare and contrast different aspects of substance use disorder such as investigation of transcripts regulated by cocaine self-administration, cocaine conditioned place preference, and the incubation of cocaine craving after prolonged withdrawal in the nucleus accumbens, several of which are freely available in the Gene Expression Omnibus database. Additionally, both strategies could be applied to other brain regions associated with substance use disorders such as the ventral tegmental area, medial prefrontal cortex, amygdala, or hippocampus. Further, these strategies could also be applied to other highly addictive substances, such as the most commonly abused drug, alcohol (Conway et al., 2006), or to compare the effects of different drugs to identify promising targets that underlie addiction to many addictive substances rather than one specific drug. If retinoic acid signaling and AKT signaling were identified through analysis of drugs other than cocaine, this would further validate these signaling pathways as important for substance use disorder in general and not just cocaine use disorder.

TRANSLATION OF BIOINFORMATIC STRATEGIES FOR ANXIETY/DEPRESSION

Either strategy could also be used to investigate other animal models of mood disorders, such as chronic social defeat stress, a model of depression. Interestingly, some rodents are resilient to developing a depression-like phenotype while others are susceptible in this paradigm (Der-Avakian et al., 2014). A future study could use Strategy 2 to examine the overlap of transcripts regulated by environmental enrichment and available transcriptomics of mice resilient to social defeat stress (Bagot et al., 2016).

Alternatively, Strategy 1 could be used to examine the effects of social defeat stress at both the transcriptomic and proteomic levels.

Additionally, these bioinformatic strategies could be used in the future to provide support for the novel inoculation stress hypothesis of environmental enrichment posited in Chapter 2. For example, Strategy 2 could be used to examine the convergence of transcripts or proteins regulated by environmental enrichment and chronic mild stress exposure. We would expect to find correspondence between the cellular effects of enrichment and chronic mild stress exposure as a literature search identified many behavioral, endocrine, physiological, as well as some neurobiological similarities between these effects (Crofton et al., 2015). These future studies would expand upon this dissertation and add to the current understanding of the resilience and susceptibility to neuropsychiatric disorders.

FUTURE USES FOR TOPOGRAPHIC TRANSCRIPTOMICS ANALYSIS

Strategy 2 compared transcriptomic data from enrichment, cocaine, as well as a topographic transcriptomic analysis of the nucleus accumbens shell. We demonstrated successful identification of genes with specific enrichment of expression in the shell over neighboring regions and manipulated one shell specific gene *in vivo*. This topographic strategy could be used in the future to identify novel targets in other brain regions. As discussed in Chapter 1, the reward circuitry involves several brain regions including the VTA, hippocampus, amygdala, and prefrontal cortex. The genome-wide *in situ* hybridization data is freely available online and could be used to investigate any of these regions. We used the correlation tool to search for NAcSh specific genes, but there is also a fine structure search tool for several different brain regions. The fine structure search tool returns a list of genes with specific expression in the region. Additionally, the Anatomic Gene Expression Atlas can be used to search for genes with enhancement in a specific seed voxel of the brain. However, future topographic analyses using the AGEA

would be improved if the AGEA was expanded to include all of the genes in the Allen Mouse Brain Atlas, rather than just those with *in situ* hybridization experiments in the coronal plane.

TARGET IDENTIFICATION AND VALIDATION

Using the two bioinformatic strategies, we identified *AKT signaling* and *RA signaling* as being promising targets involved in environmental enrichment in the nucleus accumbens. A caveat of the future use of these strategies is the bioinformatic strategies employed provide correlative evidence but not causative evidence of the role of these signaling pathways in the control of behavior. As demonstrated in this dissertation, the identified pathways must be manipulated *in vivo* to provide evidence of a causal role. Thus, we validated the target pathways underlying enrichment with *in vivo* manipulation of GSK3β and FABP5.

Translation of AKT Signaling and GSK3β as therapeutic target

AKT and GSK3β have previously been shown to be important molecules in a variety of psychiatric disorders (Beaulieu et al., 2009; Beaulieu, 2012) but this is the first examination of the role of GSK3β in cocaine self-administration as well as in combination with anxiety-like and depression-like behavioral screening. Previous studies have implicated GSK3β as downstream of dopamine receptors (Beaulieu et al., 2011; Beaulieu, 2012) as well as involved in the regulation of voltage-gated ion channels (Shavkunov et al., 2013; James et al., 2015). The current modulation of tonically active neurons in GSK3β knockdown animals warrants further investigation of the relationship between GSK3β and HCN channels as well as voltage-gated sodium channels. Future studies should focus on ion channels and other downstream targets of GSK3β. Of particular interest is the role of GSK3β in the different cell types of the nucleus accumbens shell. Unpublished data suggest GSK3β also modulates activity of NAcSh

medium spiny neurons, but it is unknown whether these alterations are due to changes in TAN modulatory activity and whether the NAcSh MSNs are primarily D1 or D2 MSNs. One strategy to investigate the role of GSK3β in these cell populations would be to express the GSK3β shRNA selectively in TANs and examine changes in neuronal activity. This could be achieved by expressing the GSK3β shRNA with a promoter specific to cholinergic neurons (e.g. choline acetyltransferase promoter). Choline acetyltransferase is an enzyme that catalyzes the synthesis of acetylcholine and according to the Allen Mouse Brain Atlas, is expressed in the mouse dorsal and ventral striatum. If the effects of the knockdown of GSK3β in the NAcSh were driven primarily by alteration in TAN activity, we would expect to see a similar increase in depression-like and addiction-related behaviors with a decrease in anxiety-like behaviors with specific knockdown of GSK3β in tonically active interneurons of the NAcSh.

Even though lithium and haloperidol inhibit GSK3β (Klein and Melton, 1996; Emamian et al., 2004), at least as part of their mechanism of action, manipulating GSK3β itself for the treatment of substance use disorders and/or mood disorders may prove difficult. We showed here that knockdown of GSK3β specifically in the NAcSh, increases cocaine self-administration and depression-like behaviors. These results suggest that GSK3β inhibition may not prove to be therapeutic and that GSK3β activity is more complicated than previously thought. Additionally, GSK3β is a ubiquitous kinase with more than 40 molecular targets and controls many different signaling pathways (Beaulieu et al., 2009), therefore, GSK3β manipulation could cause unintended side effects. Interestingly, most of the targets of GSK3β must be pre-phosphorylated before they will be phosphorylated by GSK3β and activity of GSK3β is inhibited by phosphorylation at the serine 9 site by causing the N-terminal tail to act as the pre-phosphorylated substrate and block the active site (Dajani et al., 2001). However, this inhibition of GSK3β by the N-terminal tail can be overcome by high concentrations of a substrate (Beurel et al., 2015). Therefore, rather than specifically targeting GSK3β, which is exquisitely

regulated and regulates many targets in the cell, future studies should investigate the feasibility and the therapeutic effects of disrupting the activity of GSK3β at specific targets, such as voltage-gated sodium channels, rather than the activity of GSK3β itself. Previous evidence suggests an important modulatory role of GSK3β on voltage-gated sodium channels, and blocking phosphorylation of these channels by GSK3β may prove therapeutic (Stoilova-McPhie et al., 2013; Hsu et al., 2015; James et al., 2015). Therefore, a better understanding of the intricate regulation of GSK3β and GSK3β targets may lead to effective pharmacotherapeutics for substance use disorders and mood disorders.

Translation of RA Signaling and FABP5 as therapeutic target

The target identified with Strategy 2, *RA signaling*, is particularly exciting as this is a novel target underlying environmental enrichment and addiction-related behavior. Much is unknown about the role of retinoic acid signaling in the brain. Future studies should further explore the role of retinoic acid signaling in substance use disorders and comorbid anxiety and depression.

We found that knockdown of FABP5 in the NAcSh specifically decreased cocaine-taking behaviors, which suggests that manipulation of RA signaling through inhibition of FABP5 is a promising therapeutic target for cocaine use disorder (Chapter 5). Our group has previously found that indirectly increasing retinoic acid in the NAcSh by knocking down the degradation enzyme CYP26B1 increases cocaine self-administration (Zhang et al., 2016a), an effect opposite of the FABP5 knockdown seen here. Knockdown of the other retinoic acid binding partner, CRABP2, had no effect on cocaine self-administration (unpublished data from Zhang et al. and dissertation of Y. Zhang, 2016). In order to investigate the feasibility and therapeutic potential of inhibiting FABP5 as a therapeutic target for cocaine use disorder, future studies should examine the effects of pharmacological inhibition of FABP5 on cocaine taking and seeking behaviors,

particularly the effects of inhibition of FABP5 on cocaine-induced reinstatement following prolonged cocaine withdrawal, such as through systemic injections of an inhibitor of FABP5 and FABP7, SBFI26 (Thanos et al., 2016). A reduction in cocaine-induced reinstatement after prolonged withdrawal would indicate that inhibition of FABP5 may help treatment-seeking individuals maintain abstinence after relapse to cocaine taking. Additionally, a better understanding of the role of RA signaling on cocaine–taking and –seeking behaviors may reveal other promising therapeutic targets.

Future studies should also examine the role of retinoic acid signaling in mood disorders such as anxiety and depression. Knockdown of FABP5 did not alter anxiety-like or depression-like behaviors, therefore direct inhibition of FABP5 may be translationally relevant for cocaine use disorder, but not for anxiety and depression. Therefore, a better understanding of the regulation and control of RA signaling on anxiety- and depression-like behaviors is needed. Although FABP5 does not alter anxiety-like and depression-like behaviors, RA signaling does seem to play a role in these behaviors and manipulating RA signaling through other targets modulates these behaviors. Retinoic acid has been used previously as a treatment for acne through oral administration of a derivative of retinol, isotretinoin. Isotretinoin is FDA approved for acne treatment, but has several side effects including an increased risk of depression, especially in adolescents (Azoulay et al., 2008; Layton, 2009). Additionally, our unpublished data show that knockdown of CYP26B1 decreases anxiety-like behavior and increases depression-like behavior, mimicking the susceptible phenotype of isolation (unpublished data from Zhang et al. and dissertation of Y. Zhang, 2016). On the other hand, knocking down CRABP2 mimicked the resilient depression phenotype of enrichment (unpublished data from Zhang et al. and dissertation of Y. Zhang, 2016). As discussed in Chapter 5, future studies should seek to better understand the role of retinoic acid signaling in these behaviors, especially the genomic and non-genomic effects of RA signaling via CRABP2/RARs and FABP5/PPARD, to inform future pharmacotherapeutic

development. These two separate pathways may differentially regulate the control of RA signaling on emotional behavior and drug taking behavior.

A better understanding of the role of retinoic acid signaling in the nucleus accumbens could lead to an effective pharmacotherapeutic for comorbid mood disorders and cocaine use disorder. However, the current analyses focused specifically on the nucleus accumbens and the shell region. Future analyses should investigate the role of RA signaling in the control of behavior in other brain regions in the reward circuitry and throughout the body, as a treatment would likely be systemically administered.

Novel shRNA viral vector tools developed in this dissertation

Through validation of these targets, two novel adeno-associated viral vectors were designed, constructed, and validated that use RNA interference via expression of short hairpin RNAs. These vectors can be used to examine AKT and RA signaling in other brain regions or even other tissue types in rats. Additionally, the AAV shRNA targeting GSK3β can also be used in experiments with mice or with human cell lines as the GSK3β shRNA matches mouse GSK3β and human GSK3β (via NCBI basic local alignment search tool (BLAST)). Although the shRNA sequence targeting GSK3β matches to human GSK3β, it is unlikely to be a promising therapeutic in patients with cocaine use disorder and/or mood disorders. Adeno-associated viruses have been used in clinical trials and have shown promise for both monogenetic disorders as well as Parkinson's disease (Daya and Berns, 2008). However, gene therapy with this AAV-shGSK3β vector would require highly invasive brain surgery, and if effective, may only last a year or less. However, both of these novel vectors can be used preclinically to better understand RA and AKT signaling and aid in target identification for treatment with small molecules.

OVERALL SUMMARY

Taken together, this dissertation successfully identified and validated two novel targets for comorbid cocaine use disorder and mood disorders through two bioinformatic strategies examining the nucleus accumbens of a rodent model of resilience to depression and addiction. This highlights the potential role for retinoic acid signaling and AKT signaling underlying the individual differences in susceptibility to psychiatric disorders. Future work should focus on better understanding the roles of AKT signaling and RA signaling in mood related behaviors and drug related behaviors.

References

Abelson KS, Adem B, Royo F, Carlsson HE, Hau J (2005) High plasma corticosterone levels persist during frequent automatic blood sampling in rats. In vivo (Athens, Greece) 19:815-819.

Ahmed SH, Koob GF (1997) Cocaine- but not food-seeking behavior is reinstated by stress after extinction. Psychopharmacology 132:289-295.

Ahmed SH, Koob GF (2005) Transition to drug addiction: a negative reinforcement model based on an allostatic decrease in reward function. Psychopharmacology 180:473-490.

Alcantara AA, Chen V, Herring BE, Mendenhall JM, Berlanga ML (2003) Localization of dopamine D2 receptors on cholinergic interneurons of the dorsal striatum and nucleus accumbens of the rat. Brain research 986:22-29.

Alibhai IN, Green TA, Potashkin JA, Nestler EJ (2007) Regulation of fosB and DeltafosB mRNA expression: in vivo and in vitro studies. Brain research 1143:22-33.

Allen C, Kendall JW (1967) Maturation of the circadian rhythm of plasma corticosterone in the rat. Endocrinology 80:926-930.

Alvers KM, Marusich JA, Gipson CD, Beckmann JS, Bardo MT (2012) Environmental enrichment during development decreases intravenous self-administration of methylphenidate at low unit doses in rats. Behav Pharmacol 23:650-657.

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM, Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. Nature neuroscience 6:51-58.

Anastasio NC, Stutz SJ, Fox RG, Sears RM, Emeson RB, DiLeone RJ, O'Neil RT, Fink LH, Li D, Green TA, Moeller FG, Cunningham KA (2014) Functional status of the serotonin 5-HT2C receptor (5-HT2CR) drives interlocked phenotypes that precipitate relapse-like behaviors in cocaine dependence. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39:370-382.

Anderson SM, Schmidt HD, Pierce RC (2006) Administration of the D2 dopamine receptor antagonist sulpiride into the shell, but not the core, of the nucleus accumbens attenuates cocaine priming-induced reinstatement of drug seeking. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:1452-1461.

Aosaki T, Tsubokawa H, Ishida A, Watanabe K, Graybiel AM, Kimura M (1994) Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. The Journal of neuroscience 14:3969-3984.

Aoto J, Nam CI, Poon MM, Ting P, Chen L (2008) Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. Neuron 60:308-320.

Apicella P (2002) Tonically active neurons in the primate striatum and their role in the processing of information about motivationally relevant events. European Journal of Neuroscience 16:2017-2026.

Apicella P (2007) Leading tonically active neurons of the striatum from reward detection to context recognition. Trends in neurosciences 30:299-306.

Ari Ş, Arikan M (2016) Next-Generation Sequencing: Advantages, Disadvantages, and Future. In: Plant Omics: Trends and Applications, pp 109-135: Springer.

Arroyo M, Markou A, Robbins TW, Everitt BJ (1998) Acquisition, maintenance and reinstatement of intravenous cocaine self-administration under a second-order schedule of reinforcement in rats: effects of conditioned cues and continuous access to cocaine. Psychopharmacology 140:331-344.

Aschauer DF, Kreuz S, Rumpel S (2013) Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. PloS one 8:e76310.

Azoulay L, Blais L, Koren G, LeLorier J, Berard A (2008) Isotretinoin and the risk of depression in patients with acne vulgaris: a case-crossover study. The Journal of clinical psychiatry 69:526-532.

Bagot RC et al. (2016) Circuit-wide Transcriptional Profiling Reveals Brain Region-Specific Gene Networks Regulating Depression Susceptibility. Neuron 90:969-983.

Balaraman Y, Limaye AR, Levey AI, Srinivasan S (2006) Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. Cellular and molecular life sciences : CMLS 63:1226-1235.

Balmer JE, Blomhoff R (2002) Gene expression regulation by retinoic acid. Journal of lipid research 43:1773-1808.

Bardo M, Klebaur J, Valone J, Deaton C (2001a) Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. Psychopharmacology 155:278-284.

Bardo M, Valone J, Robinet P, Shaw W, Dwoskin L (1999) Environmental enrichment enhances the stimulant effect of intravenous amphetamine: search for a cellular mechanism in the nucleus accumbens. Psychobiology 27:292-299.

Bardo MT, Bevins RA (2000) Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology 153:31-43.

Bardo MT, Klebaur JE, Valone JM, Deaton C (2001b) Environmental enrichment decreases intravenous self-administration of amphetamine in female and male rats. Psychopharmacology 155:278-284.

Bardo MT, Bowling SL, Rowlett JK, Manderscheid P, Buxton ST, Dwoskin LP (1995) Environmental enrichment attenuates locomotor sensitization, but not in vitro dopamine release, induced by amphetamine. Pharmacology, biochemistry, and behavior 51:397-405.

Barnum CJ, Blandino P, Jr., Deak T (2007) Adaptation in the corticosterone and hyperthermic responses to stress following repeated stressor exposure. J Neuroendocrinol 19:632-642.

Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC, Zachariou V, Nestler EJ (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proc Natl Acad Sci U S A 99:11435-11440.

Beaulieu JM (2012) A role for Akt and glycogen synthase kinase-3 as integrators of dopamine and serotonin neurotransmission in mental health. In: J Psychiatry Neurosci, pp 7-16.

Beaulieu JM, Gainetdinov RR, Caron MG (2009) Akt/GSK3 signaling in the action of psychotropic drugs. Annual review of pharmacology and toxicology 49:327-347.

Beaulieu JM, Del'guidice T, Sotnikova TD, Lemasson M, Gainetdinov RR (2011) Beyond cAMP: The Regulation of Akt and GSK3 by Dopamine Receptors. Frontiers in molecular neuroscience 4:38.

Belke TW (2000) Studies of wheel-running reinforcement: Parameters of Herrnstein's (1970) response-strength equation vary with schedule order. Journal of the experimental analysis of Behavior 73:319-331.

Belz EE, Kennell JS, Czambel RK, Rubin RT, Rhodes ME (2003) Environmental enrichment lowers stress-responsive hormones in singly housed male and female rats. Pharmacology Biochemistry and Behavior 76:481-486.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the royal statistical society Series B (Methodological):289-300.

Bennett BD, Wilson CJ (1999) Spontaneous activity of neostriatal cholinergic interneurons in vitro. The Journal of neuroscience 19:5586-5596.

Benzon C, Johnson S, McCue D, Li D, Green T, Hommel J (2014) Neuromedin U receptor 2 knockdown in the paraventricular nucleus modifies behavioral responses to obesogenic high-fat food and leads to increased body weight. Neuroscience 258:270-279.

Berhow MT, Hiroi N, Nestler EJ (1996) Regulation of ERK (extracellular signal regulated kinase), part of the neurotrophin signal transduction cascade, in the rat mesolimbic dopamine system by chronic exposure to morphine or cocaine. The Journal of neuroscience : the official journal of the Society for Neuroscience 16:4707-4715.

Berlanga M, Olsen C, Chen V, Ikegami A, Herring B, Duvauchelle C, Alcantara A (2003) Cholinergic interneurons of the nucleus accumbens and dorsal striatum are activated by the self-administration of cocaine. Neuroscience 120:1149-1156.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science (New York, NY) 311:864-868.

Beurel E, Grieco SF, Jope RS (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacology & therapeutics 0:114-131.

Bevins RA, Bardo MT (1999) Conditioned increase in place preference by access to novel objects: antagonism by MK-801. Behavioural brain research 99:53-60.

Bewernick BH, Hurlemann R, Matusch A, Kayser S, Grubert C, Hadrysiewicz B, Axmacher N, Lemke M, Cooper-Mahkorn D, Cohen MX (2010) Nucleus accumbens deep brain stimulation decreases ratings of depression and anxiety in treatment-resistant depression. Biological psychiatry 67:110-116.

Bezard E, Dovero S, Belin D, Duconger S, Jackson-Lewis V, Przedborski S, Piazza PV, Gross CE, Jaber M (2003) Enriched environment confers resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and cocaine: involvement of dopamine transporter and trophic factors. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:10999-11007.

Bilbao A, Rieker C, Cannella N, Parlato R, Golda S, Piechota M, Korostynski M, Engblom D, Przewlocki R, Schutz G, Spanagel R, Parkitna JR (2014) CREB activity in dopamine D1 receptor expressing neurons regulates cocaine-induced behavioral effects. Frontiers in behavioral neuroscience 8:212.

Bouet V, Freret T, Dutar P, Billard JM, Boulouard M (2011) Continuous enriched environment improves learning and memory in adult NMRI mice through theta

burst-related-LTP independent mechanisms but is not efficient in advanced aged animals. Mechanisms of ageing and development 132:240-248.

Bowling S, Bardo M (1994) Locomotor and rewarding effects of amphetamine in enriched, social, and isolate reared rats. Pharmacology Biochemistry and Behavior 48:459-464.

Bowling SL, Rowlett JK, Bardo MT (1993) The effect of environmental enrichment on amphetamine-stimulated locomotor activity, dopamine synthesis and dopamine release. Neuropharmacology 32:885-893.

Brenes JC, Rodriguez O, Fornaguera J (2008) Differential effect of environment enrichment and social isolation on depressive-like behavior, spontaneous activity and serotonin and norepinephrine concentration in prefrontal cortex and ventral striatum. Pharmacology, biochemistry, and behavior 89:85-93.

Brenes Saenz JC, Villagra OR, Fornaguera Trias J (2006) Factor analysis of Forced Swimming test, Sucrose Preference test and Open Field test on enriched, social and isolated reared rats. Behavioural brain research 169:57-65.

Bruijnzeel AW (2012) Tobacco addiction and the dysregulation of brain stress systems. Neuroscience and biobehavioral reviews 36:1418-1441.

Buono MJ, Yeager JE, Hodgdon JA (1986) Plasma adrenocorticotropin and cortisol responses to brief high-intensity exercise in humans. Journal of Applied Physiology 61:1337-1339.

Burke AR, Miczek KA (2014) Stress in adolescence and drugs of abuse in rodent models: Role of dopamine, CRF, and HPA axis. Psychopharmacology 231:1557-1580.

Butte JC, Kakihana R, Noble EP (1976) Circadian rhythm of corticosterone levels in rat brain. J Endocrinol 68:235-239.

Buwalda B, Scholte J, de Boer SF, Coppens CM, Koolhaas JM (2012) The acute glucocorticoid stress response does not differentiate between rewarding and aversive social stimuli in rats. Hormones and behavior 61:218-226.

Cachope R, Mateo Y, Mathur BN, Irving J, Wang H-L, Morales M, Lovinger DM, Cheer JF (2012) Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. Cell reports 2:33-41.

Carroll ME, Meisch RA (1984) Increased drug-reinforced behavior due to food deprivation. Advances in behavioral pharmacology 4:47-88.

Carter RN, Pinnock SB, Herbert J (2004) Does the amygdala modulate adaptation to repeated stress? Neuroscience 126:9-19.

Center NDI (2011) The Economic Impact of Illicit Drug Use on American Society. In: (Justice USDo, ed). Washington D.C.

Chauvet C, Goldberg SR, Jaber M, Solinas M (2012) Effects of environmental enrichment on the incubation of cocaine craving. Neuropharmacology 63:635-641.

Chen L, Lau AG, Sarti F (2014) Synaptic retinoic acid signaling and homeostatic synaptic plasticity. Neuropharmacology 78:3-12.

Chen X, Herbert J (1995) Regional changes in c-fos expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. Neuroscience 64:675-685.

Christiansen S, Bouzinova E, Palme R, Wiborg O (2012) Circadian activity of the hypothalamic-pituitary-adrenal axis is differentially affected in the rat chronic mild stress model of depression. Stress 15:647-657.

Chuhma N, Mingote S, Moore H, Rayport S (2014) Dopamine neurons control striatal cholinergic neurons via regionally heterogeneous dopamine and glutamate signaling. Neuron 81:901-912.

Conway KP, Compton W, Stinson FS, Grant BF (2006) Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. The Journal of clinical psychiatry.

Council NR (2011) Guide for the Care and Use of Laboratory Animals: Eighth Edition: The National Academies Press.

Covington HE, 3rd, Miczek KA (2005) Intense cocaine self-administration after episodic social defeat stress, but not after aggressive behavior: dissociation from corticosterone activation. Psychopharmacology 183:331-340.

Cox J, Mann M (2012) 1D and 2D annotation enrichment: a statistical method integrating quantitative proteomics with complementary high-throughput data. BMC bioinformatics 13 Suppl 16:S12.

Crofton EJ, Zhang Y, Green TA (2015) Inoculation stress hypothesis of environmental enrichment. Neuroscience & Biobehavioral Reviews 49:19-31.

Crofton EJ, Zhang Y, Green TA (2017a) The Proteomics of Cocaine in the Nucleus Accumbens. In: The Neuroscience of Cocaine: Mechanisms and Treatment (V.R. P, ed): Elsevier Science & Technology Books.

Crofton EJ, Nenov MN, Zhang Y, Scala F, Page SA, McCue DL, Li D, Hommel JD, Laezza F, Green TA (2017b) Glycogen synthase kinase 3 beta alters anxiety-,

depression-, and addiction-related behaviors and neuronal activity in the nucleus accumbens shell. Neuropharmacology 117:49-60.

Cruz FC, Marin MT, Leao RM, Planeta CS (2012) Behavioral and neuroendocrine effects of the exposure to chronic restraint or variable stress in early adolescent rats. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 30:19-23.

Dajani R, Fraser E, Roe SM, Young N, Good V, Dale TC, Pearl LH (2001) Crystal structure of glycogen synthase kinase 3 beta: structural basis for phosphate-primed substrate specificity and autoinhibition. Cell 105:721-732.

Dalley JW, Fryer TD, Brichard L, Robinson ES, Theobald DE, Lääne K, Peña Y, Murphy ER, Shah Y, Probst K (2007) Nucleus accumbens D2/3 receptors predict trait impulsivity and cocaine reinforcement. Science (New York, NY) 315:1267-1270.

Daly TM (2004) Overview of adeno-associated viral vectors. Gene Delivery to Mammalian Cells: Volume 2: Viral Gene Transfer Techniques:157-165.

Daya S, Berns KI (2008) Gene therapy using adeno-associated virus vectors. Clinical microbiology reviews 21:583-593.

De Boer SF, Koopmans SJ, Slangen JL, Van der Gugten J (1990) Plasma catecholamine, corticosterone and glucose responses to repeated stress in rats: effect of interstressor interval length. Physiology & behavior 47:1117-1124.

Deehan GA, Jr., Cain ME, Kiefer SW (2007) Differential rearing conditions alter operant responding for ethanol in outbred rats. Alcoholism, clinical and experimental research 31:1692-1698.

Deinzer R, Kirschbaum C, Gresele C, Hellhammer DH (1997) Adrenocortical responses to repeated parachute jumping and subsequent h-CRH challenge in inexperienced healthy subjects. Physiology & behavior 61:507-511.

Deng P, Zhang Y, Xu ZC (2007) Involvement of Ih in dopamine modulation of tonic firing in striatal cholinergic interneurons. The Journal of neuroscience 27:3148-3156.

Der-Avakian A, Mazei-Robison MS, Kesby JP, Nestler EJ, Markou A (2014) Enduring Deficits in Brain Reward Function after Chronic Social Defeat in Rats: Susceptibility, Resilience, and Antidepressant Response. Biological psychiatry.

Deroche V, Piazza PV, Deminiere JM, Le Moal M, Simon H (1993) Rats orally self-administer corticosterone. Brain research 622:315-320.

Deroche V, Piazza PV, Casolini P, Maccari S, Le Moal M, Simon H (1992) Stress-induced sensitization to amphetamine and morphine psychomotor effects depend on stress-induced corticosterone secretion. Brain research 598:343-348.

Diamond MC, Krech D, Rosenzweig MR (1964) The effects of an enriched environment on the histology of the rat cerebral cortex. The Journal of comparative neurology 123:111-120.

Dienstbier RA (1989) Arousal and physiological toughness: implications for mental and physical health. Psychological review 96:84-100.

Dietz DM et al. (2012) Rac1 is essential in cocaine-induced structural plasticity of nucleus accumbens neurons. Nature neuroscience 15:891-896.

Dobrakovova M, Kvetnansky R, Oprsalova Z, Macho L (1990) [The effect of chronic stress on the activity of the sympathetic-adrenomedullary system]. Bratislavske lekarske listy 91:587-592.

Dong Y, Green T, Saal D, Marie H, Neve R, Nestler EJ, Malenka RC (2006) CREB modulates excitability of nucleus accumbens neurons. Nature neuroscience 9:475-477.

Douglass J, Daoud S (1996) Characterization of the human cDNA and genomic DNA encoding CART: a cocaine-and amphetamine-regulated transcript. Gene 169:241-245.

Douglass J, McKinzie AA, Couceyro P (1995) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. Journal of Neuroscience 15:2471-2481.

Duester G (2008) Retinoic acid synthesis and signaling during early organogenesis. Cell 134:921-931.

Duman C, Duman R (2005) Neurobiology and treatment of anxiety: signal transduction and neural plasticity. In: Anxiety and Anxiolytic Drugs, pp 305-334: Springer.

Emamian ES, Hall D, Birnbaum MJ, Karayiorgou M, Gogos JA (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. Nature genetics 36:131-137.

English DF, Ibanez-Sandoval O, Stark E, Tecuapetla F, Buzsaki G, Deisseroth K, Tepper JM, Koos T (2012) GABAergic circuits mediate the reinforcement-related signals of striatal cholinergic interneurons. Nat Neurosci 15:123-130.

Erb S, Shaham Y, Stewart J (1996) Stress reinstates cocaine-seeking behavior after prolonged extinction and a drug-free period. Psychopharmacology 128:408-412.

Fan X, Li D, Zhang Y, Green TA (2013a) Differential phosphoproteome regulation of nucleus accumbens in environmentally enriched and isolated rats in response to acute stress. PloS one 8:e79893.

Fan X, Li D, Lichti CF, Green TA (2013b) Dynamic proteomics of nucleus accumbens in response to acute psychological stress in environmentally enriched and isolated rats. PloS one 8:e73689.

Fediuc S, Campbell JE, Riddell MC (2006) Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Dawley rats. Journal of applied physiology (Bethesda, Md : 1985) 100:1867-1875.

Felitti VJ, Anda RF, Nordenberg D, Williamson DF, Spitz AM, Edwards V, Koss MP, Marks JS (1998) Relationship of childhood abuse and household dysfunction to many of the leading causes of death in adults. The Adverse Childhood Experiences (ACE) Study. American journal of preventive medicine 14:245-258.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806-811.

Fox C, Merali Z, Harrison C (2006) Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress. Behavioural brain research 175:1-8.

Friske JE, Gammie SC (2005) Environmental enrichment alters plus maze, but not maternal defense performance in mice. Physiology & behavior 85:187-194.

Furey ML, Pietrini P, Haxby JV, Drevets WC (2008) Selective effects of cholinergic modulation on task performance during selective attention. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:913-923.

Gadek-Michalska A, Bugajski J (2003) Repeated handling, restraint, or chronic crowding impair the hypothalamic-pituitary-adrenocortical response to acute restraint stress. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 54:449-459.

Garcia-Keller C, Kupchik YM, Gipson CD, Brown RM, Spencer S, Bollati F, Esparza MA, Roberts-Wolfe DJ, Heinsbroek JA, Bobadilla AC, Cancela LM, Kalivas PW (2016) Glutamatergic mechanisms of comorbidity between acute stress and cocaine self-administration. Molecular psychiatry 21:1063-1069.

Gärtner K, Büttner D, Döhler K, Friedel R, Lindena J, Trautschold I (1980) Stress response of rats to handling and experimental procedures. Laboratory Animals 14:267-274.

Gerra G, Zaimovic A, Mascetti GG, Gardini S, Zambelli U, Timpano M, Raggi MA, Brambilla F (2001) Neuroendocrine responses to experimentally-induced psychological stress in healthy humans. Psychoneuroendocrinology 26:91-107.

Gill KE, Beveridge TJ, Smith HR, Porrino LJ (2013) The effects of rearing environment and chronic methylphenidate administration on behavior and dopamine receptors in adolescent rats. Brain research 1527:67-78.

Goeders NE (2002) Stress and cocaine addiction. The Journal of pharmacology and experimental therapeutics 301:785-789.

Goeders NE, Guerin GF (1994) Non-contingent electric footshock facilitates the acquisition of intravenous cocaine self-administration in rats. Psychopharmacology 114:63-70.

Goeders NE, Guerin GF (1996a) Effects of surgical and pharmacological adrenalectomy on the initiation and maintenance of intravenous cocaine self-administration in rats. Brain research 722:145-152.

Goeders NE, Guerin GF (1996b) Role of corticosterone in intravenous cocaine self-administration in rats. Neuroendocrinology 64:337-348.

Gormanns P, Mueller NS, Ditzen C, Wolf S, Holsboer F, Turck CW (2011) Phenome-transcriptome correlation unravels anxiety and depression related pathways. Journal of psychiatric research 45:973-979.

Graf EN, Wheeler RA, Baker DA, Ebben AL, Hill JE, McReynolds JR, Robble MA, Vranjkovic O, Wheeler DS, Mantsch JR, Gasser PJ (2013) Corticosterone acts in the nucleus accumbens to enhance dopamine signaling and potentiate reinstatement of cocaine seeking. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:11800-11810.

Green TA, Gehrke BJ, Bardo MT (2002) Environmental enrichment decreases intravenous amphetamine self-administration in rats: dose-response functions for fixed- and progressive-ratio schedules. Psychopharmacology 162:373-378.

Green TA, Crooks PA, Bardo MT, Dwoskin LP (2001) Contributory role for nornicotine in nicotine neuropharmacology: nornicotine-evoked [3 H] dopamine overflow from rat nucleus accumbens slices. Biochemical pharmacology 62:1597-1603.

Green TA, Cain ME, Thompson M, Bardo MT (2003) Environmental enrichment decreases nicotine-induced hyperactivity in rats. Psychopharmacology 170:235-241.

Green TA, Alibhai IN, Unterberg S, Neve RL, Ghose S, Tamminga CA, Nestler EJ (2008) Induction of activating transcription factors (ATFs) ATF2, ATF3, and ATF4 in the nucleus accumbens and their regulation of emotional behavior. The Journal of Neuroscience 28:2025-2032.

Green TA, Alibhai IN, Hommel JD, DiLeone RJ, Kumar A, Theobald DE, Neve RL, Nestler EJ (2006) Induction of inducible cAMP early repressor expression in nucleus accumbens by stress or amphetamine increases behavioral responses to emotional stimuli. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:8235-8242.

Green TA, Alibhai IN, Roybal CN, Winstanley CA, Theobald DE, Birnbaum SG, Graham AR, Unterberg S, Graham DL, Vialou V, Bass CE, Terwilliger EF, Bardo MT, Nestler EJ (2010) Environmental enrichment produces a behavioral phenotype mediated by low cyclic adenosine monophosphate response element binding (CREB) activity in the nucleus accumbens. Biological psychiatry 67:28-35.

Greenwood BN, Foley TE, Le TV, Strong PV, Loughridge AB, Day HE, Fleshner M (2011) Long-term voluntary wheel running is rewarding and produces plasticity in the mesolimbic reward pathway. Behavioural brain research 217:354-362.

Gregus A, Wintink AJ, Davis AC, Kalynchuk LE (2005) Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats. Behavioural brain research 156:105-114.

Grimes CA, Jope RS (2001) The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391-426.

Grimm JW, Osincup D, Wells B, Manaois M, Fyall A, Buse C, Harkness JH (2008) Environmental enrichment attenuates cue-induced reinstatement of sucrose seeking in rats. Behav Pharmacol 19:777-785.

Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proceedings of the National Academy of Sciences 97:9390-9395.

Haney M, Spealman R (2008) Controversies in translational research: drug self-administration. Psychopharmacology 199:403-419.

Haney M, Maccari S, Le Moal M, Simon H, Piazza PV (1995) Social stress increases the acquisition of cocaine self-administration in male and female rats. Brain research 698:46-52.

Hannon GJ (2002) RNA interference. Nature 418:244-251.

Hardeveld F, Spijker J, De Graaf R, Nolen WA, Beekman AT (2013) Recurrence of major depressive disorder and its predictors in the general population: results from the Netherlands Mental Health Survey and Incidence Study (NEMESIS). Psychological medicine 43:39-48.

Harlow HF (1958) The nature of love. American psychologist 13:673.

Harlow HF, Suomi SJ (1971) Social recovery by isolation-reared monkeys. Proc Natl Acad Sci U S A 68:1534-1538.

Harris RB, Gu H, Mitchell TD, Endale L, Russo M, Ryan DH (2004) Increased glucocorticoid response to a novel stress in rats that have been restrained. Physiology & behavior 81:557-568.

Hatcher JP, Bell DJ, Reed TJ, Hagan JJ (1997) Chronic mild stress-induced reductions in saccharin intake depend upon feeding status. Journal of psychopharmacology (Oxford, England) 11:331-338.

Heim C, Owens MJ, Plotsky PM, Nemeroff CB (1997) The role of early adverse life events in the etiology of depression and posttraumatic stress disorder. Focus on corticotropin-releasing factor. Annals of the New York Academy of Sciences 821:194-207.

Hennessy MB, Heybach JP, Vernikos J, Levine S (1979) Plasma corticosterone concentrations sensitively reflect levels of stimulus intensity in the rat. Physiology & behavior 22:821-825.

Hennessy MM, Weinberg J (1990) Adrenocortical activity during conditions of brief social separation in preweaning rats. Behavioral and neural biology 54:42-55.

Higley MJ, Gittis AH, Oldenburg IA, Balthasar N, Seal RP, Edwards RH, Lowell BB, Kreitzer AC, Sabatini BL (2011) Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. PLoS One 6:e19155.

Hikida T, Kitabatake Y, Pastan I, Nakanishi S (2003) Acetylcholine enhancement in the nucleus accumbens prevents addictive behaviors of cocaine and morphine. Proceedings of the National Academy of Sciences 100:6169-6173.

Hikida T, Kaneko S, Isobe T, Kitabatake Y, Watanabe D, Pastan I, Nakanishi S (2001) Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens. Proc Natl Acad Sci U S A 98:13351-13354.

Hockly E, Cordery PM, Woodman B, Mahal A, van Dellen A, Blakemore C, Lewis CM, Hannan AJ, Bates GP (2002) Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. Annals of neurology 51:235-242.

Hoeflich KP, Luo J, Rubie EA, Tsao M-S, Jin O, Woodgett JR (2000) Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation. Nature 406:86-90.

Hommel JD, Sears RM, Georgescu D, Simmons DL, DiLeone RJ (2003) Local gene knockdown in the brain using viral-mediated RNA interference. Nature medicine 9:1539-1544.

Hooper C, Killick R, Lovestone S (2008) The GSK3 hypothesis of Alzheimer's disease. J Neurochem 104:1433-1439.

Hope B, Kosofsky B, Hyman SE, Nestler EJ (1992) Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. Proc Natl Acad Sci U S A 89:5764-5768.

Hsu W-C, Nenov MN, Shavkunov A, Panova N, Zhan M, Laezza F (2015) Identifying a Kinase Network Regulating FGF14: Nav1. 6 Complex Assembly Using Split-Luciferase Complementation. PloS one 10.

Hu YS, Long N, Pigino G, Brady ST, Lazarov O (2013) Molecular mechanisms of environmental enrichment: impairments in Akt/GSK3beta, neurotrophin-3 and CREB signaling. PloS one 8:e64460.

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. Annu Rev Neurosci 29:565-598.

Ikemoto S, Panksepp J (1999) The role of nucleus accumbens dopamine in motivated behavior: a unifying interpretation with special reference to reward-seeking. Brain research Brain research reviews 31:6-41.

James TF, Nenov MN, Wildburger NC, Lichti CF, Luisi J, Vergara F, Panova-Electronova NI, Nilsson CL, Rudra JS, Green TA, Labate D, Laezza F (2015) The Na1.2 channel is regulated by GSK3. Biochimica et biophysica acta 1850:832-844.

Jankowsky JL, Xu G, Fromholt D, Gonzales V, Borchelt DR (2003) Environmental enrichment exacerbates amyloid plaque formation in a transgenic mouse model of Alzheimer disease. Journal of neuropathology and experimental neurology 62:1220-1227.

Jankowsky JL, Melnikova T, Fadale DJ, Xu GM, Slunt HH, Gonzales V, Younkin LH, Younkin SG, Borchelt DR, Savonenko AV (2005) Environmental enrichment mitigates cognitive deficits in a mouse model of Alzheimer's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5217-5224.

Jeong YH, Kim JM, Yoo J, Lee SH, Kim HS, Suh YH (2011) Environmental enrichment compensates for the effects of stress on disease progression in Tg2576 mice, an Alzheimer's disease model. J Neurochem 119:1282-1293.

Jope RS (2011) Glycogen synthase kinase-3 in the etiology and treatment of mood disorders. Frontiers in molecular neuroscience 4:16.

Jorge E, Fernandez JA, Torres R, Vergara P, Martin MT (2010) Functional changes induced by psychological stress are not enough to cause intestinal inflammation in

Sprague-Dawley rats. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 22:e241-250.

Kaczocha M, Rebecchi MJ, Ralph BP, Teng YH, Berger WT, Galbavy W, Elmes MW, Glaser ST, Wang L, Rizzo RC, Deutsch DG, Ojima I (2014) Inhibition of fatty acid binding proteins elevates brain anandamide levels and produces analgesia. PloS one 9:e94200.

Kalivas PW (2004) Glutamate systems in cocaine addiction. Current opinion in pharmacology 4:23-29.

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. American Journal of Psychiatry 162:1403-1413.

Kane MA, Folias AE, Wang C, Napoli JL (2008) Quantitative profiling of endogenous retinoic acid in vivo and in vitro by tandem mass spectrometry. Analytical chemistry 80:1702-1708.

Kehne JH, McCloskey TC, Baron BM, Chi EM, Harrison BL, Whitten JP, Palfreyman MG (1991) NMDA receptor complex antagonists have potential anxiolytic effects as measured with separation-induced ultrasonic vocalizations. European journal of pharmacology 193:283-292.

Kendler KS, Karkowski LM, Prescott CA (1998) Stressful life events and major depression: risk period, long-term contextual threat, and diagnostic specificity. The Journal of nervous and mental disease 186:661-669.

Kendler KS, Karkowski LM, Prescott CA (1999) Causal Relationship Between Stressful Life Events and the Onset of Major Depression. American Journal of Psychiatry 156:837.

Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE (2005) Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry 62:617-627.

Khoshaba DM, Maddi SR (1999) Early Experiences in Hardiness Development. Consulting Psychology Journal: Practice and Research 51:106-116.

Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209-216.

Kim WY, Shin SR, Kim S, Jeon S, Kim JH (2009) Cocaine regulates ezrin-radixin-moesin proteins and RhoA signaling in the nucleus accumbens. Neuroscience 163:501-505.

Klein PS, Melton DA (1996) A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci U S A 93:8455-8459.

Ko KW, Rasband MN, Meseguer V, Kramer RH, Golding NL (2016) Serotonin modulates spike probability in the axon initial segment through HCN channels. Nature neuroscience.

Konkle AT, Kentner AC, Baker SL, Stewart A, Bielajew C (2010) Environmental-enrichment-related variations in behavioral, biochemical, and physiologic responses of Sprague-Dawley and Long Evans rats. J Am Assoc Lab Anim Sci 49:427-436.

Koob GF, Le Moal M (2001) Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 24:97-129.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:217-238.

Koolhaas J, De Boer S, De Rutter A, Meerlo P, Sgoifo A (1996) Social stress in rats and mice. Acta physiologica scandinavica Supplementum 640:69-72.

Kosten TA, Miserendino MJ, Kehoe P (2000) Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. Brain research 875:44-50.

Kozlovsky N, Belmaker RH, Agam G (2001) Low GSK-3 activity in frontal cortex of schizophrenic patients. Schizophrenia research 52:101-105.

Kozorovitskiy Y, Gross CG, Kopil C, Battaglia L, McBreen M, Stranahan AM, Gould E (2005) Experience induces structural and biochemical changes in the adult primate brain. Proc Natl Acad Sci U S A 102:17478-17482.

Krishnan V, Nestler EJ (2011) Animal models of depression: molecular perspectives. In: Molecular and Functional Models in Neuropsychiatry, pp 121-147: Springer.

Kuhn CM, Pauk J, Schanberg SM (1990) Endocrine responses to mother-infant separation in developing rats. Developmental psychobiology 23:395-410.

Laplante F, Lappi DA, Sullivan RM (2011) Cholinergic depletion in the nucleus accumbens: effects on amphetamine response and sensorimotor gating. Progress in Neuro-Psychopharmacology and Biological Psychiatry 35:501-509.

Larson EB, Graham DL, Arzaga RR, Buzin N, Webb J, Green TA, Bass CE, Neve RL, Terwilliger EF, Nestler EJ, Self DW (2011) Overexpression of CREB in the nucleus accumbens shell increases cocaine reinforcement in self-administering rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:16447-16457.

Larsson F, Winblad B, Mohammed AH (2002) Psychological stress and environmental adaptation in enriched vs. impoverished housed rats. Pharmacology, biochemistry, and behavior 73:193-207.

Latapy C, Rioux Vàr, Guitton MJ, Beaulieu J-M (2012) Selective deletion of forebrain glycogen synthase kinase 3≈í,â§ reveals a central role in serotonin-sensitive anxiety and social behaviour. Philosophical Transactions of the Royal Society of London B: Biological Sciences 367:2460-2474.

Laviola G, Hannan AJ, Macri S, Solinas M, Jaber M (2008) Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. Neurobiology of disease 31:159-168.

Layton A (2009) The use of isotretinoin in acne. In: Dermatoendocrinol, pp 162-169.

Leal-Galicia P, Saldivar-Gonzalez A, Morimoto S, Arias C (2007) Exposure to environmental enrichment elicits differential hippocampal cell proliferation: role of individual responsiveness to anxiety. Developmental neurobiology 67:395-405.

Lehmann ML, Herkenham M (2011) Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:6159-6173.

Lein ES et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168-176.

Lenz JD, Lobo MK (2013) Optogenetic insights into striatal function and behavior. Behavioural brain research 255:44-54.

Lepsch LB, Gonzalo LA, Magro FJ, Delucia R, Scavone C, Planeta CS (2005) Exposure to chronic stress increases the locomotor response to cocaine and the basal levels of corticosterone in adolescent rats. Addict Biol 10:251-256.

Levi O, Jongen-Relo AL, Feldon J, Roses AD, Michaelson DM (2003) ApoE4 impairs hippocampal plasticity isoform-specifically and blocks the environmental stimulation of synaptogenesis and memory. Neurobiology of disease 13:273-282.

Levin FR, Bisaga A, Raby W, Aharonovich E, Rubin E, Mariani J, Brooks DJ, Garawi F, Nunes EV (2008) Effects of major depressive disorder and attention-deficit/hyperactivity disorder on the outcome of treatment for cocaine dependence. Journal of substance abuse treatment 34:80-89.

Levine S, Huchton DM, Wiener SG, Rosenfeld P (1991) Time course of the effect of maternal deprivation on the hypothalamic-pituitary-adrenal axis in the infant rat. Developmental psychobiology 24:547-558.

Li CY, Mao X, Wei L (2008) Genes and (common) pathways underlying drug addiction. PLoS computational biology 4:e2.

Lichti CF, Fan X, English RD, Zhang Y, Li D, Kong F, Sinha M, Andersen CR, Spratt H, Luxon BA, Green TA (2014) Environmental enrichment alters protein expression as well as the proteomic response to cocaine in rat nucleus accumbens. Frontiers in behavioral neuroscience 8:246.

Lim SAO, Kang UJ, McGehee DS (2014) Striatal cholinergic interneuron regulation and circuit effects. Frontiers in synaptic neuroscience 6:22.

Lobo MK, Covington HE, 3rd, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, Dietz DM, Zaman S, Koo JW, Kennedy PJ, Mouzon E, Mogri M, Neve RL, Deisseroth K, Han MH, Nestler EJ (2010) Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. Science (New York, NY) 330:385-390.

Lobo MK et al. (2013) DeltaFosB induction in striatal medium spiny neuron subtypes in response to chronic pharmacological, emotional, and optogenetic stimuli. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:18381-18395.

Louilot A, Le Moal M, Simon H (1986) Differential reactivity of dopaminergic neurons in the nucleus accumbens in response to different behavioral situations. An in vivo voltammetric study in free moving rats. Brain research 397:395-400.

Lyons DM, Parker KJ, Schatzberg AF (2010) Animal models of early life stress: Implications for understanding resilience. Developmental psychobiology 52:402-410.

Lyons DM, Parker KJ, Katz M, Schatzberg AF (2009) Developmental cascades linking stress inoculation, arousal regulation, and resilience. Frontiers in behavioral neuroscience 3:32.

Ma L, Hasan KM, Steinberg JL, Narayana PA, Lane SD, Zuniga EA, Kramer LA, Moeller FG (2009) Diffusion tensor imaging in cocaine dependence: regional effects of cocaine on corpus callosum and effect of cocaine administration route. Drug and alcohol dependence 104:262-267.

MacDougall MJ, Howland JG (2013) Acute stress, but not corticosterone, disrupts short- and long-term synaptic plasticity in rat dorsal subiculum via glucocorticoid receptor activation. Cerebral cortex (New York, NY : 1991) 23:2611-2619.

Maden M (2007) Retinoic acid in the development, regeneration and maintenance of the nervous system. Nature Reviews Neuroscience 8:755-765.

Magarinos AM, McEwen BS (1995) Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors. Neuroscience 69:83-88.

Maghsoodi B, Poon MM, Nam CI, Aoto J, Ting P, Chen L (2008) Retinoic acid regulates RARalpha-mediated control of translation in dendritic RNA granules during homeostatic synaptic plasticity. Proc Natl Acad Sci U S A 105:16015-16020.

Maier T, Guell M, Serrano L (2009) Correlation of mRNA and protein in complex biological samples. FEBS letters 583:3966-3973.

Malenka RC, Nestler E, Hyman S, Sydor A, Brown R (2009) Molecular Neuropharmacology: A Foundation for Clinical Neuroscience. New York: McGrawHill Medical Book.

Manning CE, Williams ES, Robison AJ (2017) Reward Network Immediate Early Gene Expression in Mood Disorders. Frontiers in behavioral neuroscience 11.

Marti O, Gavalda A, Jolin T, Armario A (1993) Effect of regularity of exposure to chronic immobilization stress on the circadian pattern of pituitary adrenal hormones, growth hormone, and thyroid stimulating hormone in the adult male rat. Psychoneuroendocrinology 18:67-77.

Matthews K, Wilkinson LS, Robbins TW (1996) Repeated maternal separation of preweanling rats attenuates behavioral responses to primary and conditioned incentives in adulthood. Physiology & behavior 59:99-107.

McClung CA, Nestler EJ (2003) Regulation of gene expression and cocaine reward by CREB and DeltaFosB. Nature neuroscience 6:1208-1215.

McCormick CM, Kehoe P, Kovacs S (1998) Corticosterone release in response to repeated, short episodes of neonatal isolation: evidence of sensitization. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 16:175-185.

McEwen BS (2000) Allostasis and allostatic load: implications for neuropsychopharmacology. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 22:108-124.

McEwen BS, Wingfield JC (2010) What is in a name? Integrating homeostasis, allostasis and stress. Hormones and behavior 57:105-111.

McKinzie D, RODD-HENRICKS Z, Dagon C, Murphy J, McBride W (1999) Cocaine Is Self-administered into the Shell Region of the Nucleus Accumbens in Wistar Rats. Annals of the New York Academy of Sciences 877:788-791.

McMahon LR, Cunningham KA (1999) Antagonism of 5-hydroxytryptamine4 receptors attenuates hyperactivity induced by cocaine: putative role for 5-hydroxytryptamine4 receptors in the nucleus accumbens shell. Journal of Pharmacology and Experimental Therapeutics 291:300-307.

Meichenbaum D (2007) Stress Inoculation Training: A Preventative and Treatment Approach. In: Principles and Practice of Stress Management, 3 Edition (Lehrer PM, Woolfolk RL, Sime WS, eds): Guilford Press.

Merali Z, Levac C, Anisman H (2003) Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice. Biological psychiatry 54:552-565.

Meredith GE, Baldo BA, Andrezjewski ME, Kelley AE (2008) The structural basis for mapping behavior onto the ventral striatum and its subdivisions. Brain Structure and Function 213:17-27.

Miczek KA, Mutschler NH (1996) Activational effects of social stress on IV cocaine self-administration in rats. Psychopharmacology 128:256-264.

Miller JS, Tallarida RJ, Unterwald EM (2009) Cocaine-induced hyperactivity and sensitization are dependent on GSK3. Neuropharmacology 56:1116-1123.

Miller JS, Tallarida RJ, Unterwald EM (2010) Inhibition of GSK3 attenuates dopamine D1 receptor agonist-induced hyperactivity in mice. Brain Res Bull 82:184-187.

Miller JS, Barr JL, Harper LJ, Poole RL, Gould TJ, Unterwald EM (2014) The GSK3 signaling pathway is activated by cocaine and is critical for cocaine conditioned reward in mice. PloS one 9:e88026.

Miyata K, Kamato T, Nishida A, Ito H, Yuki H, Yamano M, Tsutsumi R, Katsuyama Y, Honda K (1992) Role of the serotonin3 receptor in stress-induced defecation. Journal of Pharmacology and Experimental Therapeutics 261:297-303.

Mizoguchi K, Yuzurihara M, Ishige A, Sasaki H, Chui DH, Tabira T (2001) Chronic stress differentially regulates glucocorticoid negative feedback response in rats. Psychoneuroendocrinology 26:443-459.

Mlynarik M, Johansson BB, Jezova D (2004) Enriched environment influences adrenocortical response to immune challenge and glutamate receptor gene expression in rat hippocampus. Annals of the New York Academy of Sciences 1018:273-280.

Moffett MC, Harley J, Francis D, Sanghani SP, Davis WI, Kuhar MJ (2006) Maternal separation and handling affects cocaine self-administration in both the treated pups as adults and the dams. The Journal of pharmacology and experimental therapeutics 317:1210-1218.

Moldow RL, Fischman AJ (1987) Cocaine induced secretion of ACTH, beta-endorphin, and corticosterone. Peptides 8:819-822.

Moncek F, Duncko R, Johansson BB, Jezova D (2004) Effect of environmental enrichment on stress related systems in rats. In: J Neuroendocrinol, pp 423-431. England.

Morris G, Arkadir D, Nevet A, Vaadia E, Bergman H (2004) Coincident but distinct messages of midbrain dopamine and striatal tonically active neurons. Neuron 43:133-143.

Morris MC, Ciesla JA, Garber J (2010) A prospective study of stress autonomy versus stress sensitization in adolescents at varied risk for depression. Journal of abnormal psychology 119:341-354.

Morris MC, Rao U, Garber J (2012) Cortisol responses to psychosocial stress predict depression trajectories: Social-evaluative threat and prior depressive episodes as moderators. Journal of affective disorders 143:223-230.

Mortimer JT, Staff J (2004) Early work as a source of developmental discontinuity during the transition to adulthood. Development and psychopathology 16:1047-1070.

Muller MB, Landgraf R, Preil J, Sillaber I, Kresse AE, Keck ME, Zimmermann S, Holsboer F, Wurst W (2000) Selective activation of the hypothalamic vasopressinergic system in mice deficient for the corticotropin-releasing hormone receptor 1 is dependent on glucocorticoids. Endocrinology 141:4262-4269.

Murison R, Hansen AL (2001) Reliability of the chronic mild stress paradigm: implications for research and animal welfare. Integrative Physiological & Behavioral Science 36:266-274.

Nader J, Claudia C, El Rawas R, Favot L, Jaber M, Thiriet N, Solinas M (2012a) Loss of environmental enrichment increases vulnerability to cocaine addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:1579-1587.

Nader J, Chauvet C, Rawas RE, Favot L, Jaber M, Thiriet N, Solinas M (2012b) Loss of environmental enrichment increases vulnerability to cocaine addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:1579-1587.

Natelson BH, Ottenweller JE, Cook JA, Pitman D, McCarty R, Tapp WN (1988) Effect of stressor intensity on habituation of the adrenocortical stress response. Physiology & behavior 43:41-46.

Nenov MN, Tempia F, Denner L, Dineley KT, Laezza F (2015) Impaired firing properties of dentate granule neurons in an Alzheimer's disease animal model are rescued by PPARgamma agonism. Journal of neurophysiology 113:1712-1726.

Nestler EJ (2001) Molecular basis of long-term plasticity underlying addiction. Nature reviews neuroscience 2:119-128.

Nestler EJ (2005) The Neurobiology of Cocaine Addiction. Science & Practice Perspectives.

Nestler EJ (2015) Role of the Brain's Reward Circuitry in Depression: Transcriptional Mechanisms. International review of neurobiology 124:151-170.

Nestler EJ, Carlezon WA, Jr. (2006) The mesolimbic dopamine reward circuit in depression. Biological psychiatry 59:1151-1159.

Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nature neuroscience 13:1161-1169.

Nestler EJ, Gould E, Manji H (2002) Preclinical models: status of basic research in depression. Biological psychiatry 52:503-528.

Ng L, Bernard A, Lau C, Overly CC, Dong HW, Kuan C, Pathak S, Sunkin SM, Dang C, Bohland JW, Bokil H, Mitra PP, Puelles L, Hohmann J, Anderson DJ, Lein ES, Jones AR, Hawrylycz M (2009) An anatomic gene expression atlas of the adult mouse brain. Nature neuroscience 12:356-362.

Niewiadomska-Cimicka A, Krzyżosiak A, Ye T, Podleśny-Drabiniok A, Dembélé D, Dollé P, Krężel W (2016) Genome-wide Analysis of RARβ Transcriptional Targets in Mouse Striatum Links Retinoic Acid Signaling with Huntington's Disease and Other Neurodegenerative Disorders. Molecular neurobiology:1-20.

Nwaneshiudu CA, Unterwald EM (2010) NK-3 receptor antagonism prevents behavioral sensitization to cocaine: a role of glycogen synthase kinase-3 in the nucleus accumbens. J Neurochem 115:635-642.

O'Brien WT, Harper AD, Jove F, Woodgett JR, Maretto S, Piccolo S, Klein PS (2004) Glycogen synthase kinase-3beta haploinsufficiency mimics the behavioral and molecular effects of lithium. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:6791-6798.

O'Connor EC, Chapman K, Butler P, Mead AN (2011) The predictive validity of the rat self-administration model for abuse liability. Neuroscience & Biobehavioral Reviews 35:912-938.

Ortinski PI, Briand LA, Pierce RC, Schmidt HD (2015) Cocaine-seeking is associated with PKC-dependent reduction of excitatory signaling in accumbens shell D2 dopamine receptor-expressing neurons. Neuropharmacology 92C:80-89.

Ostrander MM, Ulrich-Lai YM, Choi DC, Richtand NM, Herman JP (2006) Hypoactivity of the hypothalamo-pituitary-adrenocortical axis during recovery from chronic variable stress. Endocrinology 147:2008-2017.

Pan B, Zhong P, Sun D, Liu QS (2011) Extracellular signal-regulated kinase signaling in the ventral tegmental area mediates cocaine-induced synaptic plasticity and rewarding effects. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:11244-11255.

Pardo M, King MK, Perez-Costas E, Melendez-Ferro M, Martinez A, Beurel E, Jope RS (2015) Impairments in cognition and neural precursor cell proliferation in mice expressing constitutively active glycogen synthase kinase-3. Frontiers in behavioral neuroscience 9.

Parker KJ, Maestripieri D (2011) Identifying key features of early stressful experiences that produce stress vulnerability and resilience in primates. Neuroscience & Biobehavioral Reviews 35:1466-1483.

Parker KJ, Buckmaster CL, Schatzberg AF, Lyons DM (2004) Prospective investigation of stress inoculation in young monkeys. Archives of general psychiatry 61:933-941.

Pavlovsky AA, Boehning D, Li D, Zhang Y, Fan X, Green TA (2013) Psychological stress, cocaine and natural reward each induce endoplasmic reticulum stress genes in rat brain. Neuroscience 246:160-169.

Pellow S, File SE (1986) Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacology Biochemistry and Behavior 24:525-529.

Pena Y, Prunell M, Rotllant D, Armario A, Escorihuela RM (2009) Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. Psychoneuroendocrinology 34:1390-1404.

Penn E, Tracy DK (2012) The drugs don't work? Antidepressants and the current and future pharmacological management of depression. Therapeutic advances in psychopharmacology 2:179-188.

Perrine SA, Miller JS, Unterwald EM (2008) Cocaine regulates protein kinase B and glycogen synthase kinase-3 activity in selective regions of rat brain. J Neurochem 107:570-577.

Perrotti LI, Hadeishi Y, Ulery PG, Barrot M, Monteggia L, Duman RS, Nestler EJ (2004) Induction of deltaFosB in reward-related brain structures after chronic stress. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:10594-10602.

Pettinati HM, O'Brien CP, Dundon WD (2013) Current status of co-occurring mood and substance use disorders: a new therapeutic target. The American journal of psychiatry 170:23-30.

Piazza PV, Le Moal M (1998) The role of stress in drug self-administration. Trends in pharmacological sciences 19:67-74.

Piazza PV, Deminiere JM, le Moal M, Simon H (1990) Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. Brain research 514:22-26.

Piazza PV, Maccari S, Deminière JM, Le Moal M, Mormède P, Simon H (1991) Corticosterone levels determine individual vulnerability to amphetamine self-administration. Proc Natl Acad Sci U S A 88:2088-2092.

Pisani A, Bernardi G, Ding J, Surmeier DJ (2007) Re-emergence of striatal cholinergic interneurons in movement disorders. Trends in neurosciences 30:545-553.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr. (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:7397-7403.

Pohorecky LA (1991) Stress and alcohol interaction: an update of human research. Alcoholism, clinical and experimental research 15:438-459.

Pontieri FE, Tanda G, Di Chiara G (1995) Intravenous cocaine, morphine, and amphetamine preferentially increase extracellular dopamine in the "shell" as compared with the "core" of the rat nucleus accumbens. Proc Natl Acad Sci U S A 92:12304-12308.

Puhl MD, Blum JS, Acosta-Torres S, Grigson PS (2012) Environmental enrichment protects against the acquisition of cocaine self-administration in adult male rats, but does not eliminate avoidance of a drug-associated saccharin cue. Behav Pharmacol 23:43-53.

Rabasa C, Delgado-Morales R, Munoz-Abellan C, Nadal R, Armario A (2011a) Adaptation of the hypothalamic-pituitary-adrenal axis and glucose to repeated immobilization or restraint stress is not influenced by associative signals. Behavioural brain research 217:232-239.

Rabasa C, Munoz-Abellan C, Daviu N, Nadal R, Armario A (2011b) Repeated exposure to immobilization or two different footshock intensities reveals differential adaptation of the hypothalamic-pituitary-adrenal axis. Physiology & behavior 103:125-133.

Ranaldi R, Kest K, Zellner M, Hachimine-Semprebom P (2011) Environmental enrichment, administered after establishment of cocaine self-administration, reduces lever pressing in extinction and during a cocaine context renewal test. Behav Pharmacol 22:347-353.

Raz S (2013) Ameliorative effects of brief daily periods of social interaction on isolation-induced behavioral and hormonal alterations. Physiology & behavior 116-117:13-22.

Rebec GV, Grabner CP, Johnson M, Pierce RC, Bardo MT (1997) Transient increases in catecholaminergic activity in medial prefrontal cortex and nucleus accumbens shell during novelty. Neuroscience 76:707-714.

Renner MJ, Rosenzweig MR (1987) Enriched and Impoverished Environments: Effects on Brain and Behavior. New York: Springer.

Retana-Marquez S, Bonilla-Jaime H, Velazquez-Moctezuma J (1998) Lack of effect of corticosterone administration on male sexual behavior of rats. Physiology & behavior 63:367-370.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004a) Rational siRNA design for RNA interference. Nature biotechnology 22:326-330.

Reynolds JN, Hyland BI, Wickens JR (2004b) Modulation of an afterhyperpolarization by the substantia nigra induces pauses in the tonic firing of striatal cholinergic interneurons. The Journal of neuroscience 24:9870-9877.

Robinson TE, Berridge KC (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. Brain research reviews 18:247-291.

Rojas Vega S, Strüder HK, Vera Wahrmann B, Schmidt A, Bloch W, Hollmann W (2006) Acute BDNF and cortisol response to low intensity exercise and following ramp incremental exercise to exhaustion in humans. Brain research 1121:59-65.

Rosenzweig MR (2003) Effects of Differential Experience on the Brain and Behavior. Developmental Neuropsychology 24:523-540.

Rosenzweig MR, Bennett EL (1996) Psychobiology of plasticity: effects of training and experience on brain and behavior. Behavioural brain research 78:57-65.

Roy V, Belzung C, Delarue C, Chapillon P (2001) Environmental enrichment in BALB/c mice: effects in classical tests of anxiety and exposure to a predatory odor. Physiology & behavior 74:313-320.

Russo SJ, Nestler EJ (2013) The brain reward circuitry in mood disorders. Nature reviews Neuroscience 14.

Rutter M (2006) Implications of resilience concepts for scientific understanding. Annals of the New York Academy of Sciences 1094:1-12.

Schroeder JP, Spanos M, Stevenson JR, Besheer J, Salling M, Hodge CW (2008) Cue-induced reinstatement of alcohol-seeking behavior is associated with increased ERK1/2 phosphorylation in specific limbic brain regions: blockade by the mGluR5 antagonist MPEP. In: Neuropharmacology, pp 546-554. England.

Schug TT, Berry DC, Shaw NS, Travis SN, Noy N (2007) Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell 129:723-733.

Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115:199-208.

Seery MD, Leo RJ, Lupien SP, Kondrak CL, Almonte JL (2013) An upside to adversity?: moderate cumulative lifetime adversity is associated with resilient responses in the face of controlled stressors. Psychological science 24:1181-1189.

Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ (1998) Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:1848-1859.

Selye H (1976) Stress without distress. In: Psychopathology of Human Adaptation (Serban G, ed), pp 137-146. New York: Springer.

Shaham Y, Stewart J (1994) Exposure to mild stress enhances the reinforcing efficacy of intravenous heroin self-administration in rats. Psychopharmacology 114:523-527.

Shaham Y, Shalev U, Lu L, de Wit H, Stewart J (2003) The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology 168:3-20.

Sharp JL, Zammit TG, Azar TA, Lawson DM (2002) Stress-like responses to common procedures in male rats housed alone or with other rats. Contemporary topics in laboratory animal science / American Association for Laboratory Animal Science 41:8-14.

Shavkunov AS, Wildburger NC, Nenov MN, James TF, Buzhdygan TP, Panova-Elektronova NI, Green TA, Veselenak RL, Bourne N, Laezza F (2013) The fibroblast growth factor 14.voltage-gated sodium channel complex is a new target of glycogen synthase kinase 3 (GSK3). The Journal of biological chemistry 288:19370-19385.

Shi X, Miller JS, Harper LJ, Poole RL, Gould TJ, Unterwald EM (2014) Reactivation of cocaine reward memory engages the Akt/GSK3/mTOR signaling pathway and can be disrupted by GSK3 inhibition. Psychopharmacology 231:3109-3118.

Shoji H, Mizoguchi K (2010) Acute and repeated stress differentially regulates behavioral, endocrine, neural parameters relevant to emotional and stress response in young and aged rats. Behavioural brain research 211:169-177.

Sih A (2011) Effects of early stress on behavioral syndromes: an integrated adaptive perspective. Neuroscience and biobehavioral reviews 35:1452-1465.

Sinha R (2001) How does stress increase risk of drug abuse and relapse? Psychopharmacology 158:343-359.

Sinha R, Catapano D, O'Malley S (1999) Stress-induced craving and stress response in cocaine dependent individuals. Psychopharmacology 142:343-351.

Sinha R, Garcia M, Paliwal P, Kreek MJ, Rounsaville BJ (2006) Stress-induced cocaine craving and hypothalamic-pituitary-adrenal responses are predictive of cocaine relapse outcomes. Archives of general psychiatry 63:324-331.

Skwara AJ, Karwoski TE, Czambel RK, Rubin RT, Rhodes ME (2012) Influence of environmental enrichment on hypothalamic-pituitary-adrenal (HPA) responses to single-dose nicotine, continuous nicotine by osmotic mini-pumps, and nicotine withdrawal by mecamylamine in male and female rats. Behavioural brain research 234:1-10.

Smith JK, Neill JC, Costall B (1997) Post-weaning housing conditions influence the behavioural effects of cocaine and d-amphetamine. Psychopharmacology 131:23-33.

Smith MA, Iordanou JC, Cohen MB, Cole KT, Gergans SR, Lyle MA, Schmidt KT (2009) Effects of environmental enrichment on sensitivity to cocaine in female rats: importance of control rates of behavior. Behavioural pharmacology 20:312.

Solinas M, Thiriet N, Chauvet C, Jaber M (2010) Prevention and treatment of drug addiction by environmental enrichment. Prog Neurobiol 92:572-592.

Solinas M, Chauvet C, Thiriet N, El Rawas R, Jaber M (2008) Reversal of cocaine addiction by environmental enrichment. Proceedings of the National Academy of Sciences 105:17145-17150.

Solinas M, Thiriet N, El Rawas R, Lardeux V, Jaber M (2009) Environmental enrichment during early stages of life reduces the behavioral, neurochemical, and molecular effects of cocaine. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:1102-1111.

Spires TL, Grote HE, Varshney NK, Cordery PM, van Dellen A, Blakemore C, Hannan AJ (2004) Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:2270-2276.

Stairs DJ, Bardo MT (2009) Neurobehavioral effects of environmental enrichment and drug abuse vulnerability. Pharmacology Biochemistry and Behavior 92:377-382.

Stairs DJ, Klein ED, Bardo MT (2006) Effects of environmental enrichment on extinction and reinstatement of amphetamine self-administration and sucrose-maintained responding. Behavioural pharmacology 17:597-604.

Stairs DJ, Prendergast MA, Bardo MT (2011) Environmental-induced differences in corticosterone and glucocorticoid receptor blockade of amphetamine self-administration in rats. Psychopharmacology 218:293-301.

Stambolic V, Ruel L, Woodgett JR (1996) Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Current biology : CB 6:1664-1668.

Stoilova-McPhie S, Ali S, Laezza F (2013) Protein-Protein Interactions as New Targets for Ion Channel Drug Discovery. Austin J Pharmacol Ther 1.

Sturm V, Lenartz D, Koulousakis A, Treuer H, Herholz K, Klein JC, Klosterkötter J (2003) The nucleus accumbens: a target for deep brain stimulation in obsessive–compulsive-and anxiety-disorders. Journal of chemical neuroanatomy 26:293-299.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America 102:15545-15550.

Summerford C, Samulski RJ (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. Journal of virology 72:1438-1445.

Sutton LP, Rushlow WJ (2012) The dopamine D2 receptor regulates Akt and GSK-3 via Dvl-3. International Journal of Neuropsychopharmacology 15:965-979.

Swanson HH, van de Poll NE (1983) Effects of an isolated or enriched environment after handling on sexual maturation and behaviour in male and female rats. Journal of reproduction and fertility 69:165-171.

Swendsen JD, Merikangas KR (2000) The comorbidity of depression and substance use disorders. Clinical psychology review 20:173-189.

Takatsu-Coleman AL, Patti CL, Zanin KA, Zager A, Carvalho RC, Borcoi AR, Ceccon LM, Berro LF, Tufik S, Andersen ML, Frussa-Filho R (2013) Short-term social isolation induces depressive-like behaviour and reinstates the retrieval of an aversive task: mood-congruent memory in male mice? Journal of psychiatry & neuroscience : JPN 38:259-268.

Tennant C (2001) Work-related stress and depressive disorders. Journal of psychosomatic research 51:697-704.

Thanos PK, Clavin BH, Hamilton J, O'Rourke JR, Maher T, Koumas C, Miao E, Lankop J, Elhage A, Haj-Dahmane S (2016) Examination of the addictive and Behavioral

Properties of Fatty acid-Binding Protein inhibitor sBFi26. Frontiers in psychiatry 7.

Thiel KJ, Sanabria F, Pentkowski NS, Neisewander JL (2009) Anti-craving effects of environmental enrichment. The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 12:1151-1156.

Thiel KJ, Pentkowski NS, Peartree NA, Painter MR, Neisewander JL (2010) Environmental living conditions introduced during forced abstinence alter cocaine-seeking behavior and Fos protein expression. Neuroscience 171:1187-1196.

Torres G, Rivier C (1992) Cocaine-induced stimulation of the rat hypothalamic-pituitary-adrenal axis is progressively attenuated following hourly-interval regimens of the drug. Life sciences 51:1041-1048.

Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, Mann M, Cox J (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nature methods 13:731-740.

Urakawa S, Mitsushima D, Shimozuru M, Sakuma Y, Kondo Y (2014) An enriched rearing environment calms adult male rat sexual activity: implication for distinct serotonergic and hormonal responses to females. PloS one 9:e87911.

Urakawa S, Takamoto K, Hori E, Sakai N, Ono T, Nishijo H (2013) Rearing in enriched environment increases parvalbumin-positive small neurons in the amygdala and decreases anxiety-like behavior of male rats. BMC neuroscience 14:13.

Urs NM, Snyder JC, Jacobsen JP, Peterson SM, Caron MG (2012) Deletion of GSK3beta in D2R-expressing neurons reveals distinct roles for beta-arrestin signaling in antipsychotic and lithium action. Proc Natl Acad Sci U S A 109:20732-20737.

van Dellen A, Blakemore C, Deacon R, York D, Hannan AJ (2000) Delaying the onset of Huntington's in mice. Nature 404:721-722.

Van Praag H, Kempermann G, Gage FH (2000) Neural Consequences of Environmental Enrichment.

Vassalli G, Bueler H, Dudler J, von Segesser LK, Kappenberger L (2003) Adeno-associated virus (AAV) vectors achieve prolonged transgene expression in mouse myocardium and arteries in vivo: a comparative study with adenovirus vectors. International journal of cardiology 90:229-238.

Vialou V, Maze I, Renthal W, LaPlant QC, Watts EL, Mouzon E, Ghose S, Tamminga CA, Nestler EJ (2010) Serum response factor promotes resilience to chronic social stress through the induction of DeltaFosB. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:14585-14592.

Viau V, Sharma S, Meaney MJ (1996) Changes in plasma adrenocorticotropin, corticosterone, corticosteroid-binding globulin, and hippocampal glucocorticoid receptor occupancy/translocation in rat pups in response to stress. J Neuroendocrinol 8:1-8.

Wallace DL, Vialou V, Rios L, Carle-Florence TL, Chakravarty S, Kumar A, Graham DL, Green TA, Kirk A, Iniguez SD (2008) The influence of ≈í√ÆFosB in the nucleus accumbens on natural reward-related behavior. The Journal of Neuroscience 28:10272-10277.

Wallace DL, Han MH, Graham DL, Green TA, Vialou V, Iniguez SD, Cao JL, Kirk A, Chakravarty S, Kumar A, Krishnan V, Neve RL, Cooper DC, Bolanos CA, Barrot M, McClung CA, Nestler EJ (2009) CREB regulation of nucleus accumbens excitability mediates social isolation-induced behavioral deficits. Nature neuroscience 12:200-209.

Warner-Schmidt JL, Schmidt EF, Marshall JJ, Rubin AJ, Arango-Lievano M, Kaplitt MG, Ibanez-Tallon I, Heintz N, Greengard P (2012) Cholinergic interneurons in the nucleus accumbens regulate depression-like behavior. Proc Natl Acad Sci U S A 109:11360-11365.

Wildburger NC, Laezza F (2012) Control of neuronal ion channel function by glycogen synthase kinase-3: new prospective for an old kinase. Frontiers in molecular neuroscience 5:80.

Wilkinson MB, Dias C, Magida J, Mazei-Robison M, Lobo M, Kennedy P, Dietz D, Covington H, 3rd, Russo S, Neve R, Ghose S, Tamminga C, Nestler EJ (2011) A novel role of the WNT-dishevelled-GSK3beta signaling cascade in the mouse nucleus accumbens in a social defeat model of depression. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:9084-9092.

Williams MJ, Adinoff B (2008) The role of acetylcholine in cocaine addiction. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:1779-1797.

Willner P (1997) Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology 134:319-329.

Willner P (2005) Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology 52:90-110.

Willner P, Muscat R, Papp M (1992) Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neuroscience and biobehavioral reviews 16:525-534.

Willner P, Towell A, Sampson D, Sophokleous S, Muscat R (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology 93:358-364.

Wilson C, Chang H, Kitai S (1990) Firing patterns and synaptic potentials of identified giant aspiny interneurons in the rat neostriatum. The Journal of neuroscience 10:508-519.

Wilson CJ (2005) The mechanism of intrinsic amplification of hyperpolarizations and spontaneous bursting in striatal cholinergic interneurons. Neuron 45:575-585.

Witten IB, Lin S-C, Brodsky M, Prakash R, Diester I, Anikeeva P, Gradinaru V, Ramakrishnan C, Deisseroth K (2010) Cholinergic interneurons control local circuit activity and cocaine conditioning. Science (New York, NY) 330:1677-1681.

Wolf SA, Kronenberg G, Lehmann K, Blankenship A, Overall R, Staufenbiel M, Kempermann G (2006) Cognitive and physical activity differently modulate disease progression in the amyloid precursor protein (APP)-23 model of Alzheimer's disease. Biological psychiatry 60:1314-1323.

Worley MJ, Trim RS, Roesch SC, Mrnak-Meyer J, Tate SR, Brown SA (2012) Comorbid depression and substance use disorder: longitudinal associations between symptoms in a controlled trial. Journal of substance abuse treatment 43:291-302.

Wright RL, Conrad CD (2008) Enriched environment prevents chronic stress-induced spatial learning and memory deficits. Behavioural brain research 187:41-47.

Xu CM, Wang J, Wu P, Zhu WL, Li QQ, Xue YX, Zhai HF, Shi J, Lu L (2009) Glycogen synthase kinase 3beta in the nucleus accumbens core mediates cocaine-induced behavioral sensitization. J Neurochem 111:1357-1368.

Yue Y, Widmer DA, Halladay AK, Cerretti DP, Wagner GC, Dreyer JL, Zhou R (1999) Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:2090-2101.

Zakharova E, Starosciak A, Wade D, Izenwasser S (2012) Sex differences in the effects of social and physical environment on novelty-induced exploratory behavior and cocaine-stimulated locomotor activity in adolescent rats. Behavioural brain research 230:92-99.

Zarate CA, Mathews DC, Furey ML (2013) Human biomarkers of rapid antidepressant effects. Biological psychiatry 73:1142-1155.

Zhang W, Li F, Nie L (2010) Integrating multiple ‘omics’ analysis for microbial biology: application and methodologies. Microbiology 156:287-301.

Zhang Y (2016) Transcriptomics of environmental enrichment and cocaine reveals roles for retinoic acid signaling and DeltaFosB in addiction. Dissertation: The University of Texas Medical Branch.

Zhang Y, Crofton EJ, Li D, Fan X, Rudenko G, Green TA Tonic inhibition model of deltaFosB. In preparation.

Zhang Y, Crofton EJ, Li D, Lobo MK, Fan X, Nestler EJ, Green TA (2014) Overexpression of DeltaFosB in nucleus accumbens mimics the protective addiction phenotype, but not the protective depression phenotype of environmental enrichment. Frontiers in Behavioral Neuroscience 8.

Zhang Y, Kong F, Crofton EJ, Dragosljvich SN, Sinha M, Li D, Fan X, Koshy S, Hommel JD, Spratt HM (2016a) Transcriptomics of Environmental Enrichment Reveals a Role for Retinoic Acid Signaling in Addiction. Frontiers in molecular neuroscience 9.

Zhang Y, Crofton EJ, Fan X, Li D, Kong F, Sinha M, Luxon BA, Spratt HM, Lichti CF, Green TA (2016b) Convergent transcriptomics and proteomics of environmental enrichment and cocaine identifies novel therapeutic strategies for addiction. Neuroscience 339:254-266.

Zhang Y CE, Koshy S, Li D, Green TA Manipulation of retinoic acid signaling pathway in the nucleus accumbens shell changes emotional behavior in rats. In preparation.

Zhou W, Chen L, Paul J, Yang S, Li F, Sampson K, Woodgett JR, Beaulieu JM, Gamble KL, Li X (2012) The effects of glycogen synthase kinase-3beta in serotonin neurons. PloS one 7:e43262.

Vita

Elizabeth Joy Crofton was born in Phoenix, AZ on June 12, 1990 to Stephen Ernest Crofton and Karen Lynn (Brandsness) Crofton. She grew up in Paradise Valley, AZ and completed her high school education at Saguaro High School in Scottsdale, Arizona in 2008. That same year she began her tenure at St. Olaf College in Northfield, MN from which she ultimately earned her Bachelor of Arts degree in Psychology with a concentration in Neuroscience in May of 2012. After graduating from St. Olaf, she joined the Neuroscience Graduate Program at The University of Texas Medical Branch in Galveston, TX. She joined the laboratory of Dr. Thomas A. Green in the Spring of 2013 and defended her dissertation July 11, 2017.

BIBLIOGRAPHY:

URL for all publications:
https://scholar.google.com/citations?user=gqiTZkcAAAAJ&hl=en

ARTICLES IN PEER-REVIEWED JOURNALS:

Crofton EJ, Nenov MN, Zhang Y, Scala F, Page SA, Li D, Hommel JD, Laezza F, Green TA. (2017). Glycogen synthase kinase 3 beta alters anxiety-, depression, and addiction-related behaviors and neuronal activity in the nucleus accumbens shell. *Neuropharmacology*. 117, 49-60. Doi: 10.1016/j.neuropharm.2017.01.020.

Zhang Y*, Crofton EJ*, Fan X, Li D, Kong F, Sinha M, Luxon BA, Spratt HM, Lichti CF, Green TA. (2016). Convergent transcriptomics and proteomics of environmental enrichment and cocaine identifies novel therapeutic strategies for addiction. *Neuroscience*. 339, 254-266. ***These authors contributed equally.** Doi: 10.1016/j.neuroscience.2016.09.051.

Zhang Y, Kong F, **Crofton EJ**, Sinha M, Li D, Fan X, Hommel JD, Spratt HM, Luxon BA, Green TA. (2016). Transcriptomics of environmental enrichment reveals a role for retinoic acid signaling in addiction. *Frontiers in Molecular Neuroscience*, 9, 119. Doi: 10.3389/fnmol.2016.00119

Crofton EJ, Zhang Y, Green TA. (2015). Inoculation stress model of environmental enrichment, *Neuroscience and Biobehavioral Reviews.* 49c: 19-31. Doi: 10.1016/j.neubiorev.2014.11.017

Zhang Y, **Crofton EJ**, Li D, Fan X, Nestler EJ, Green TA. (2014). Overexpression of DeltaFosB in nucleus accumbens mimics the protective addiction phenotype, but not the protective depression phenotype of environmental enrichment. *Frontiers in Behavioral Neuroscience.* 8. 297. Doi: 10.3389/fnbeh.2014.00297

BOOK CHAPTER:

Crofton EJ, Zhang Y, Green TA. The proteomics of cocaine in the nucleus accumbens (2017), In Preedy (Ed.), *The Neuroscience of Cocaine: Mechanisms and Treatment*. ISBN: 9780128037508.

IN PROGRESS:

Crofton EJ*, Nenov MN*, Zhang Y*, Koshy S, Laezza F, Green TA. Topographical transcriptomics of the nucleus accumbens shell: Retinoic acid signaling. ***These authors contributed equally** (in preparation)

Scala F*, Nenov MN*, **Crofton EJ,** James TF, Alshammari M, Alshammari T, Wildburger N, Chesson BC, Lichti C, Zhang Y, D'Ascenzo M, Rudra J, Green TA, Laezza F. A GSK3-Nav1.6 axis controls functional properties of medium spiny neurons through direct phosphorylation of voltage-gated Nav1.6 channels. *These authors contributed equally (in preparation)

Zhang Y, **Crofton EJ,** Koshy S, Li D, Green TA. Manipulation of retinoic acid signaling pathway in the nucleus accumbens shell changes emotional behavior in rats (submitted)

Zhang Y, **Crofton EJ,** Li D, Rudenko G, Green TA. Tonic inhibition model of deltaFosB (in preparation)

ABSTRACTS:

Crofton EJ, Nenov MN, Zhang Y, Li D, Hommel JD, Laezza F, Green TA. Glycogen synthase kinase 3 beta in the nucleus accumbens shell alters addiction and depression behavior and reduces neuronal activity of tonically active neurons. Abstract (*Poster*), Neuroscience and Cell Biology Research Retreat, 2016

Crofton EJ, Zhang Y, Koshy S, TA Green. Topographic gene expression analysis of the nucleus accumbens shell and ventral tegmental area yields novel therapeutic target possibilities for anxiety, depression, and drug addiction. Abstract (*Poster*), Annual Meeting of the Society for Neuroscience, San Diego, 2016

Scala F, Nenov MN, **Crofton EJ**, Zhang Y, Wildburger N, Chesson B, James T, Alshammari M, Alshammari T, Lichti C, Rudra J, D'Ascenzo M, Green TA, Laezza F. GSK3 Signaling regulates intrinsic firing and persistent sodium current in medium spiny neurons of the nucleus accumbens. Abstract (*Poster*), Annual Meeting of the Society for Neuroscience, San Diego, 2016

Crofton EJ, Zhang Y, Kong F, Luxon B, Spratt H, Lichti CF, Green TA. Comparative analysis of transcriptomics and proteomics of environmental enrichment and cocaine. Abstract (*Poster*), Annual Meeting of the College on Problems of Drug Dependence, Palm Springs, CA, 2016

Zhang Y, **Crofton EJ**, Koshy S, Green TA. Crabp2 and Fabp5 mediated retinoic acid signaling is a novel mechanism controlling depression- and addiction-related behavior. Abstract (*Poster)*, Annual Meeting of the College on Problems of Drug Dependence, Palm Springs, CA, 2016

Zhang Y, **Crofton EJ**, Li D, Koshy S, Hommel JD, Green TA. Crabp2 and Fabp5 mediated retinoic acid signaling is a novel mechanism of environmental enrichment. Abstract (*Poster)*, Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX, 2016

Crofton EJ, Nenov MN, Zhang Y, Li D, Hommel JD, Laezza F, Green TA. Glycogen synthase kinase 3 beta in the nucleus accumbens shell affects neuronal activity and alters addiction and depression behavior. Abstract (*Poster*), 56th Annual National Student Research Forum, Galveston, TX, 2015

Zhang Y, **Crofton E J**, Kong F. Spratt HM, Sinha M, Andersen CR, Li D, Fan X, Luxon BA, Hommel JD, Green TA. Retinoic acid signaling is a novel mechanism of environmental enrichment. Abstract (*Poster*), 56th Annual National Student Research Forum, Galveston, TX, 2015

Crofton EJ, Zhang Y, Nenov MN, Li D, Hommel JD, Laezza F, Green TA. Glycogen Synthase Kinas 3 beta (GSK3Beta) in the Nucleus Accumbens Shell Affects Neuronal Firing and Alters Addiction and Depression Behavior. Abstract (*Poster*), 3rd Annual Clinical & Translational Research Forum, Galveston, TX, 2015

Crofton EJ, Nenov MN, Zhang Y, Fan X, Li D, Laezza F, Green TA. GSK3B silencing in the NAc produces addiction- and depression-like phenotypes opposite of environmental enrichment and alters neuronal excitability. Abstract (*Poster*), Annual Meeting of the College on Problems of Drug Dependence, Phoenix, AZ, 2015

Zhang Y, **Crofton EJ**, Li D, Fan X, Green TA. Retinoic acid signaling in the nucleus accumbens: a novel mechanism controlling addiction-related behavior.

Abstract (*Poster*), Annual Meeting of the College on Problems of Drug Dependence, Phoenix, AZ, 2015

Crofton EJ, Zhang Y, Nenov MN, Dingge Li, Laezza F, Green TA. Specific knockdown of glycogen synthase kinase 3 beta (GSK3β) in the nucleus accumbens decreases neuronal excitability and alters behavior. Abstract (*Oral Presentation*), Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX, 2015

Zhang Y, **Crofton E J**, Kong F. Spratt HM, Sinha M, Andersen CR, Li D, Fan X, Luxon BA, Hommel JD, Green TA. Retinoic acid signaling is a novel mechanism of environmental enrichment. Abstract (*Poster*), Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX, 2015

Nenov MN, Zhang Y**, Crofton EJ**, Scala F, D'Ascenzo M, Green TA, Laezza F. Environmental enrichment paradigm identifies GSK3 as a target gene for protection against psychiatric disorders. Abstract (*Poster*), 53rd Annual Meeting of the American College of Neuropsychopharmacology (ACNP), Phoenix, AZ, 2014

Crofton EJ, Zhang Y, Fan X, Li D, Green TA. Inoculation stress model of environmental enrichment. Abstract (*Poster*), Annual Meeting of the Society for Neuroscience, Washington, DC, 2014

Crofton EJ, Zhang Y, Li D, Green T. Role of NR4A1 (Nur77) and NR4A3 (NOR-1) in the nucleus accumbens in conferring the protective addiction phenotype from environmental enrichment. Abstract (*Poster*), Annual Meeting of the College on Problems of Drug Dependence, San Juan, Puerto Rico, 2014

Zhang Y, **Crofton EJ**, Li D, Fan X, Lobo MK, Nestler EJ, Green TA. Overexpression of ΔFosB in rat nucleus accumbens shell mimics environmental enrichment in sucrose self-administration and cocaine seeking. Abstract (*Poster*), Annual Meeting of the College on Problems of Drug Dependence, San Juan, Puerto Rico, 2014

Crofton E, Zhang Y, Li D, Green TA. The role of NR4A1 (Nur77) & NR4A3 (NOR-1) in environmental enrichment in the nucleus accumbens of rats. Abstract (*Poster*), 55th Annual National Student Research Forum, Galveston, TX, 2014

Crofton EJ, Zhang Y, Li D, Green TA (2014) Environmental enrichment and the role of NR4A1 (Nur77) and NR4A3 (NOR-1) in rat nucleus accumbens. Abstract (*Poster*), Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX, 2014

Bittar A, **Crofton E**, Neugebauer V (2013) Cognitive performance is inversely correlated with pain behaviors in rats. Abstract (*Poster*) presented by A Bittar, Annual Meeting of the Society for Neuroscience, 2013

Dittmann A, **Crofton E**, Raitt C, Stolp-Smith C, McMillan DK (2011) Extraversion and Extreme Response Style. Abstract (*Poster*), St. Olaf College Science Symposium*,* Northfield, MN, 2011

AWARDS:

2017 Center for Addiction Research Outstanding Student Award, Second Annual Neuroscience Graduate Program Student Symposium, The University of Texas Medical Branch

2017 Student Service Award from the Neuroscience Graduate Program, Second Annual Neuroscience Graduate Program Student Symposium, The University of Texas Medical Branch

2017 The George Sealy Research Award in Neurology, for significant contributions to the knowledge of neurology through research, The University of Texas Medical Branch in Galveston, TX

2016 Best Poster Award- Second Place at the Neuroscience and Cell Biology Research Retreat

2016 University Federal Credit Union Award for outstanding performance in all areas of academic life at The University of Texas Medical Branch

2016 Dr. and Mrs. Seymour Fisher Excellence Award in Neuroscience for the best paper submitted by a graduate student from the University of Texas Medical Branch in Galveston, TX

2016 Lynch Addiction Research Fund Travel Award to attend the Annual Meeting of the College on Problems of Drug Dependence, Palm Springs, CA

2016 Honoree in *Who's Who Among Students in American Universities and Colleges* for recognition of merit and accomplishment as a student at The University of Texas Medical Branch

2015 Ann Anderson Scholarship Award to an outstanding graduate student from the University of Texas Medical Branch in Galveston, TX

2015 Society for Neuroscience Chapter of the Year Award, Society for Neuroscience National Organization (*Secretary of SfN Chapter during 2014-2015 school year*)

2015 Overall Outstanding Poster Presentation, 56th Annual National Student Research Forum, Galveston, TX

2015 Excellence in Translational Science Award, 56th Annual National Student Research Forum, Galveston, TX

2015 Award for Best Presentation in Pharmacology and Toxicology, 56th Annual National Student Research Forum, Galveston, TX

2015 NIDA Director's Travel Award for the Annual Meeting of the College on Problems of Drug Dependence, Phoenix, AZ

2015 Travel Award for the Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX

2014 Bohdan Nechay Endowment Award to an outstanding graduate student from the University of Texas Medical Branch in Galveston, TX

2014 Travel Award for the Annual Meeting of Behavior, Biology, and Chemistry: Translational Research in Addiction, San Antonio, TX

GRANTS/FELLOWSHIPS:

Neural and Pharmacological Mechanisms of Abused Drugs
T32, DA007287, UTMB Center for Addiction Research Sept 2014- Aug 2016

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

March 2014 – Present	*The College on Problems of Drug Dependence (CPDD)* Member-In-Training
Jan 2013 – Present	*Society for Neuroscience* Member
Aug 2012 – Present	*Galveston Chapter of the Society for Neuroscience* Member
Aug 2012 – Present	*Graduate Student Organization, UTMB* Member
Aug 2008 – May 2012	*St. Olaf Psychology Club* St. Olaf College, Northfield, MN

REVIEW EXPERIENCE:

Ad hoc reviewer: Physiology and Behavior

Behavioural Brain Research
Neuroscience Letters
Psychoneuroendocrinology

MENTORING EXPERIENCE:

M.D. students:

Steven N. Dragosljvich, M.D. (UTMB)

Ph.D. students:

Luke Bourner (UTMB)
Jessica Di Re (UTMB)
Christina Merritt (UTMB)
Cynthia Tapia (UTMB)
Kathleen Winters (UTMB)

Undergraduate students:

Sean A. Page (California State, Fullerton)
Jesus Jurado (UT El Paso)

TEACHING EXPERIENCE:

The University of Texas Medical Branch, Galveston, TX

Post-Baccalaureate Research Education Program (PREP):
Critical Reading of Scientific Literature (BBSC 6104) Spring 2016 & 2017
16-week course, one credit hour, co-instructor

Cell Biology Graduate Program:
Cellular & Molecular Mechanisms in Health & Disease (CELL6401) Summer 2016
1-week guest lecturer

Institute for Medical Humanities:
Ethics of Scientific Research (MEHU 6101) Summer 2016, Fall 2016, & Spring 2017
2-day course, small group co-leader

School of Medicine First Year Curriculum:
Neuroscience and Human Behavior (IMC 1220) Spring 2015
5-week intensive neuroanatomy laboratory assistant

VOLUNTEER SERVICE AND LEADERSHIP:

April 2017- June 2017 *Chair*
Neuroscience Graduate Student Symposia Planning Committee
The University of Texas Medical Branch at Galveston

May 2014 – Aug 2015 *Executive Secretary*
Society for Neuroscience Galveston Chapter
The University of Texas Medical Branch at Galveston

May 2014 – May 2015	*Executive Secretary* Graduate Student Organization The University of Texas Medical Branch at Galveston
April 11, 2015	*Volunteer* UTMB United to Serve 2015
May 2013 – May 2014	*Graduate Student Representative* Welcome Weekend Planning Committee The University of Texas Medical Branch at Galveston
Sept 2013 – Sept 2014	*Pre-Candidacy Student Representative* Neuroscience Graduate Program The University of Texas Medical Branch at Galveston
April 12, 2014	*Volunteer* UTMB United to Serve 2014
Apr 2012 – May 2014	*Scientific Outreach Coordinator* Graduate Student Organization The University of Texas Medical Branch at Galveston
Feb 8, 2014 & Feb 21, 2015	*Category Judge* Galveston County Science and Engineering Fair University of Texas Medical Branch at Galveston, Texas A&M University at Galveston, and Galveston College

GRADUATE ROTATIONS:

Center for Addiction Research, Department of Pharmacology and Toxicology, *The University of Texas Medical Branch at Galveston*
Advisor for research rotation: Kathryn Cunningham, Ph.D.
Field of Study: Drug addiction and behavior

Department of Neuroscience and Cell Biology*, The University of Texas Medical Branch at Galveston*
Advisor for research rotation: Volker Neugebauer, M.D., Ph.D.
Field of Study: Affective Disorders

UNDERGRADUATE RESEARCH:

Neurosurgery Center for Research, Training, & Education, *Loma Linda University*, Loma Linda, CA
Advisor: Wolff M. Kirsch, M.D.
Field of Study: Alzheimer's disease

Department of Psychology, *St. Olaf College*, Northfield, MN
Advisor: Gary M. Muir, Ph.D.
Field of Study: The Neural Basis of Navigation

Department of Psychology, *St. Olaf College,* Northfield, MN
Advisor: Donna McMillan, Ph.D.
Field of Study: Extraversion

This dissertation was typed by Elizabeth Crofton.