THE COMPLEX INTERPLAY OF HEROIN, REWARD, AND GENE EXPRESSION

A Dissertation in

Neuroscience

by

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ABSTRACT

Addiction is a disease of brain reward circuitry whereby systems which evolved to respond to “life-giving” rewards (such as food and social relationships) are now primed to bring about the ultimate goal of seeking and acquiring more drug. Moreover, individual propensities to becoming addicted to a given drug vary greatly, a phenomenon that we now know finds its root in individual internal and external environments. Therefore, the primary goal of this thesis was to examine the intersection of gene-expression, environment and individual susceptibilities to addiction-like behavior. In Chapter 2, we utilize a model of reward devaluation and heroin self-administration. When the opportunity to self-administer heroin is preceded by the opportunity to ingest a sweet saccharin solution, an outbred group of Sprague-Dawley rats splits into two distinct behavioral phenotypes: Those that show a preference for saccharin and low/moderate intake of heroin (small saccharin suppressors; SS) and those that avoid the saccharin cue and escalate heroin intake (large saccharin suppressors; LS). We examined the gene expression of the corticotropin releasing factor (CRF) pathway in reward areas (medial prefrontal cortex; mPFC, hippocampus, ventral tegmental area; VTA and nucleus accumbens; NAc) of these two different phenotypes and found that LS demonstrated greater gene expression of the CRF signaling pathway across reward regions. DNA methylation analysis revealed that the LS group had decreased methylation of cytosines in the CRFbp gene promoter. Chapter 3 explored whether saccharin suppression, brought about by passive infusion of heroin, would recapitulate the gene expression findings of the self-administration model. Overall, gene expression differences were markedly different from those reported in Chapter 2, indicating that passive infusion and self-administration bring about different molecular effects. Chapter 4 investigated gene expression differences between the mPFC transcriptome of SS and LS rats. Genes found to be differentially expressed between groups included those involved in schizophrenia and dopamine signaling, signal transduction,
development and synaptic plasticity. The occurrence of several failed confirmatory qRT-PCR experiments led us to the hypothesis that the two mPFC hemispheres may not always have the same levels of gene expression. Chapter 5 explored this question through an RNA-Seq study on the left and right mPFC of drug-naïve male rats. 2.6% of expressed genes were found to differ between the left and right mPFC. Ingenuity Pathway Analysis revealed these differentially expressed genes to be involved in pathways such as cell signaling and cell morphology. Given the reports of functional laterality of the mPFC in the stress response, we asked whether the CRF pathway would be differentially expressed in reward regions in response to the passive heroin infusion model (which likely contains a stress component). CRF binding protein and CRFR2 were differentially expressed between left and right hippocampus between groups, CRF was differentially expressed between left and right VTA. Lastly, in Chapter 6, we show that environmental enrichment attenuates drug-seeking and motivation and is accompanied by expression differences of the early response genes EGR1 and EGR2. Bisulfite amplicon sequencing revealed site-specific methylation differences for EGR1 and EGR2 in the mPFC and NAc. Taken together, we show that individual propensities to drug-taking behavior are tied to differential gene expression and regulation and can be influenced by environmental factors. Moreover, we show that consideration should be given to hemisphere when investigating neurogenetic phenomena.
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ABREVIATIONS

Adora2a: Adenosine receptor 2a
Adora2b: Adenosine receptor 2b
Amyg: Amygdala
Arhgef28: Rho guanine nucleotide exchange factor 28
BCat: Beta catenin
Bche: Butecholine esterase
BLA: Basolateral amygdala
BSAS: BiSulfite Amplicon Sequencing
Cdk5: Cyclin dependent kinase 5
CeA: Central nucleus of the amygdala
ChrnB3: Nicotinic acetylcholine subunit beta 3
CORT: Corticosterone
CRF: Corticotropin Releasing Factor (transcript and protein)
CRFbp: Corticotropin Releasing Factor binding protein
CRFR1: Corticotropin Releasing Factor Receptor type 1
CRFR2: Corticotropin Releasing Factor Receptor type 2
CRH: Corticotropin Releasing Hormone (gene)
DA: Dopamine
DNA: Deoxyribonucleic acid
E/R: Extinction and reinstatement
Fxyd6: FXYD domain containing ion transport regulator 6
Grap: GRB2-related adaptor protein
HspB1: Heat-shock protein beta 1
Igf2: Insulin-like growth factor 2
Igfbp2: Insulin-like growth factor binding protein 2
IP: Intra-peritoneal (injection)
ICV: intracerebroventricular (infusion)
M: Maintenance phase
mPFC: Medial prefrontal cortex
NAc: Nucleus accumbens
NR3C1: Glucocorticoid receptor type II
Nurp1: Nuclear protein 1
OPRM1: Opiod receptor mu 1
Pcdhga5: Protocadherin gamma subfamily A,5
Pou3fa: POU domain, class 3, transcription factor 4
PR: Progressive ratio challenge test
Ptgds: Prostaglandin D2 synthase
Ptgfr: Prostaglandin F receptor
Rgs4: Regulator of G protein signaling (gene)
Rims3: Regulating synaptic membrane exocytosis 3
RNA: Ribonucleic Acid
RNA-Seq: Ribonucleic acid sequencing
SA: Self-administration
Sema3f: Semaphorin 3F
Slc13a4: Solute Carrier Family 13 (Sodium/Sulphate Symporters), Member 4
Slc6a13: Solute Carrier Family 6 (Neurotransmitter Transporter, GABA), Member 13
Slc6a20: Solute Carrier Family 6 (Proline IMINO Transporter), Member 20
Sned1: Sushi, nidogen and EGF-like domains 1
Thbs2: Thrombospondin2

Trpv1: Transient receptor potential cation channel subfamily V member 4

VTA: Ventral tegmental area

qRT-PCR: Quantitative real-time polymerase chain reaction

Zfp775: Zinc finger protein 775
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-Gerald Holton

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Chapter 1

Introduction: The Physiological and Neurological Effects of Heroin Use and Addiction

The Heroin Epidemic

History

Opium is obtained from the resin of the scored oriental opium poppy seedpod. Since prehistoric times, it has been cultivated and utilized for its analgesic and mood-enhancing properties. The Ebers Papyrus (c1500 BCE) illustrates using the strained pulp of the poppy to quiet a crying child. Early surgeries used sponges soaked in opium, *spongia somnifera*, to lessen discomfort and pain. In The Odyssey, Homer describes an opium preparation that is given to Telemachus and his friends to help them cope with the absence of Odysseus (Brownstein, 1993).

During the Eighth century, opium was brought to India and China, and between the tenth and thirteenth century, opium began to make its way into Europe. While opium was still used for its analgesic properties for conditions such as cancer, tetanus, and menstrual and childbirth pain, it was also widely used as a recreational drug, and reports began to surface of its addictive properties (Merry, 1975). In 1805, morphine was isolated from opium, and named after the Greek god of dreams, Morpheus. With 10 times the euphoric effects of opium and given the advent of the hypodermic needle, morphine was used for minor surgeries, post-operative, and chronic pain (Brownstein, 1993), and shockingly enough, to treat opium addiction (Merry, 1975). In 1875, English chemist, C. R. Alder Wright synthesized heroin by boiling anhydrous morphine with acetic anhydride. The result was an acetylated form of morphine called diacetylmorphine, morphine
diacetate or diamorphine. This compound only became widely used after it was independently synthesized by Felix Hoffman at the Bayer pharmaceutical company in Germany. The head of the Bayer research department named diamorphine “heroin” based on the German word *heroisch* meaning “heroic, strong”. From 1898-1910, heroin was marketed as a non-addictive substitute and cough suppressant by the Bayer company. Eventually, it was recognized as highly addictive, and in 1924, the US government banned its sale, importation and manufacture (Askwith, 1998).

The first heroin epidemic in the United States occurred after World War II, and largely involved young black men and the jazz scene in Chicago. At the time, high-quality and inexpensive heroin was readily available, and often was mixed with other drugs such as cocaine and cannabis. Reports also indicate that new users of heroin were not aware of its addictive properties. The decline in this first epidemic appears to be due to the rising cost of heroin, as the price of heroin doubled by 1950 (Hughes *et al*, 1972).

The second U.S. heroin epidemic was revealed when, in 1971, congressmen Robert Steele of Connecticut and Morgan Murphy of Illinois returned from a visit to Vietnam. While there, they had found that 15-20% of servicemen reported being actively addicted to heroin. President Richard Nixon created a new office, The Special Action Office of Drug Abuse Prevention, in order to design a program of prevention and rehabilitation. The head of this new office, Jerome Jaffe, enlisted the help of a psychiatric researcher named Lee Robins, to help study the addiction epidemic among the servicemen. Under the new program, soldiers that were addicted to heroin were not allowed to return home until they were clean. Once they were home, the lives of the men were followed by Robins. Surprisingly, she found that once home, only 5% of those who had previously been addicted to heroin resumed heroin use, a strong demonstration of the power of environment to influence behavior (Spiegel, 2012).
Current Heroin/Opiate Epidemic

Over the past 20 years, heroin use and addiction have been dramatically on the rise. In fact, the number of people that meet the criteria for heroin dependence in the Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM IV) doubled from 2002 to 2012 (Administration, 2013). This rise in heroin use is largely attributed to the wide-spread use and distribution of prescription opioids. A growing number of reports indicate that many people first became dependent on opioids from a prescription that was given to relieve pain (e.g., post-operative, injuries, etc.) and then turned to the cheaper and more available heroin when the prescription or money ran out (Cicero et al, 2014; Peavy et al, 2012). In fact, what used to be an inner-city problem has now become the bane of wealthy, mostly white, suburbs. A recent study that examined various parametrics of opioid use from 1960-2010 found several striking trends: 1) Of those that first began opioid use in 1960, >80% of participant’s first opioid of use was heroin; by 2000, that trend had nearly reversed with >70% of participants reporting that the first use of an opioid was from a prescription. 2) Of those that began opioid use in 1960, >80% were men; by 2010, the percentages were equal for women and men. 3) Of those who began opioid use in 1960 and 1970, nearly equivalent numbers were white and non-white; of those that began opioid use in 2010, ~90% were white (Cicero et al, 2014).

Recently, the FDA has supported the development of “abuse-deterrent” opiates; opiates with properties that combat expected routes of delivery, such as crushing in order to snort the opiate, or extraction with the intention of intravenous injection (Administration, 2017; Graham, 2016). Other pharmaceutical strategies include the release of the opioid antagonist, naltrexone, when the medication is damaged, medications that are an irritant to nasal passages should the drug be snorted and pro-drug formulations that are only activated by enzymes in the gastrointestinal tract (Moorman-Li et al, 2012). The efficacy of this strategy may not actually be “abuse-deterrent”,
and, instead, may be pushing already addicted individuals toward seeking out the easily obtained and easy to use (but unknown purity) heroin, instead. In fact, statements from interviews from the participants in the Cicero et al. (2014) study provide striking insight into the nature of the prescription opiate to heroin transition. One participant stated:

“It [OxyContin] was getting harder and harder to get the pills that you could use in a needle; most of them would just ‘gel-up.’ And it was cheaper and easier to get heroin [sic], which was much stronger and would get you higher than Oxycodone.”

Another survey respondent had a similar story:

“Heroin is cheaper and stronger than the prescription drugs listed, and the supply is typically pretty consistent. It is also much easier to use intravenously than pills and other prescriptions, which often take more complex methods to break down.” (Cicero et al, 2014).

The growing opiate addiction crisis is a significant burden on society at the micro and macro levels. Approximately $484 billion is spent on addiction related costs in the U.S. annually - more than cancer ($171.6 billion) and diabetes ($131.7 billion) combined. Costs include health care, lost earnings, crimes and traffic accidents (NIDA, 2017). In fact, it is estimated that 10-22% of all traffic accidents involve the use of drugs (including alcohol) (Administration, 2013). The cost to families and children is perhaps more sobering; the U.S. Department of Health and Human services estimates that 50-80% of child abuse or neglect cases involves parental substance abuse (services, 1994).

**Addiction Mechanisms**

**Reward Pathway**

The brain reward pathway is comprised of several brain structures that are activated in response to and in anticipation of a salient stimulus. This pathway is present in all vertebrates and evolved to
aid the survival of the organism, survival of the species and generational genetic transmission. To summarize, this pathway is activated in response to palatable stimuli such as highly caloric or nutrient rich food, positive social interactions, life or career achievement, and sex, for example.

The reward pathway (also known as the mesocorticolimbic dopamine (DA) system) is characterized by dopaminergic transmission from neurons originating in the ventral tegmental area (VTA) to several limbic brain structures; most notably, the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), amygdala and hippocampus (Figure 1-1). DA is released from the VTA in response to emotionally or motivationally salient stimuli in order to: (1) bring about adaptive behaviors and (2) facilitate synaptic plasticity that establishes learned associations regarding that event (Jay, 2003). Importantly, DA will continue to be released in the presence of a conditioned stimulus, even after the association has been learned. Thus, it also has the role of being a predictor of an impending salient event (Schultz, 1998). Each of the structures within the reward pathway has distinct and interactive roles that bring about behaviors involved in seeking and obtaining reward.

**NAc**

The NAc (also known as the ventral striatum) is an area involved in action selection, habit formation and motivation. As a part of the basal ganglia, the NAc has dense GABAergic projections to the ventral pallidum; a connection that links motivation and action (Wu *et al*, 1993). Accordingly, the NAc mediates the rewarding and motivating effects of drugs of abuse (Volkow *et al*, 2003). The NAc also receives substantial glutamatergic input from the PFC; a connection that facilitates action in the presence of drug-associated stimuli (Kalivas and Volkow, 2005). On the basis of histological, neurochemical and functional differences, the NAc is divided into two
regions: 1) the core, which is involved in motor function, and 2) the shell, which contributes more to limbic functions (Heimer et al, 1997).

**mPFC**

The medial prefrontal cortex (mPFC) is a region that receives substantial projections from the hippocampus, VTA, anterior insular areas, and amygdala, and is known for its role in decision-making, attention, and executive control, as well as orchestrating the retrieval of recent and distant memories and the consolidation of memory. The mPFC has projections to the amygdala, hippocampus, dorsal and medial striatum, dorsal raphe, ventral tegmental area, hypothalamus and locus coeruleus. Given it’s numerous connections and functions, perhaps a more comprehensive description of the mPFC is that it is a region that makes associations between context, memory and emotional content surrounding a stimulus and, therefore, selects the best action or response at a given time (Euston et al, 2012).

**Hippocampus**

The hippocampus is a brain structure most known for its role in learning and memory. Of note, it is one of the few regions in the adult human brain that is still making new neurons. In fact, it is thought that newly “born” neurons in the dentate gyrus are nourished or fed by new memories and experiences. In addition to memory consolidation, the hippocampus also plays a strong role in negative regulation of the hypothalamic pituitary adrenal axis (HPA axis), a component of the autonomic stress response. Circulating cortisol/corticosterone (CORT) crosses the blood brain barrier and binds to the plentiful glucocorticoid receptors (GRs) within the hippocampus, thus
activating an inhibitory signal to the hypothalamus, where the HPA axis originates (Jacobson and Sapolsky, 1991).

**Amygdala**

The amygdala is involved in assigning emotional significance or salience to a stimulus, and linking that salience with memory, motivation, action and attention through its widespread connections with the hippocampus, DA cells of the VTA, NAc and mPFC (Everitt *et al.*, 2003). Moreover, the Amygdala functions in orchestrating endocrine and autonomic effects via interaction with the hypothalamus and brainstem (Cardinal *et al.*, 2002).

**The Extended Amygdala**

The extended amygdala is increasingly becoming recognized for its distinct morphological and functional characteristics. The extended amygdala is comprised of the central nucleus of the amygdala as well as the lateral bed nucleus of the stria terminalis and a small portion of the caudal NAc shell. With dense reciprocal connections with the lateral hypothalamus, and DA cells of the VTA, as well as motor areas, the extended amygdala is associated with mediating the response to environmental and internal stressors (Alheid, 2003).

Obviously, all of these regions are important in reward procurement as 1) motivation and action is necessary for physically obtaining a reward, 2) attention is required to see the task to completion, 3) the organism must *feel* as though the reward is worth the effort, and 4) it is important to remember the context (e.g., place, cues) in which the reward can be obtained. The mPFC, amygdala and hippocampus also send major glutamatergic projections to the NAc, which then sends GABAergic projections to the VTA and the dorsomedial nucleus of the thalamus. The
dorsomedial nucleus of the thalamus then projects to areas of the prefrontal cortex involved in association and memory (Heimer, 2003) (Figure 1-2).
Figure 1-1. Dopaminergic transmission through the reward pathway in the rat brain. Red=Dopamine, Blue=GABA.

Figure 1-2. Glutamatergic transmission through the reward pathway in the rat brain. Black=Glutamate, Blue=GABA.
Anyone who has ever fallen in love can understand what it is to have the reward pathway stimulated to a considerable extent. As Marie Coulson said, “Falling in love is like leaping from a cliff. Your brain screams that it’s not a good idea and that hurt and pain will inevitably come to you. But your heart believes you can soar, glide and fly”. You may feel as if you are going crazy. Your mind may be consumed by thoughts of your beloved. You may neglect other important engagements for the reward of seeing that special person, you would risk nearly anything for him/her: because this person feels like life, itself. This is the power of the reward pathway over us. It was designed/evolved to keep us alive. This is the nature of addiction: seeking and obtaining drug feels like life itself.

Drugs of abuse produce pleasurable experiences because they exert their effects by stimulating this same pathway. The problem, however, stems from the magnitude of stimulation. The reward pathway is uniquely sensitive to the value, or salience, of a reward. That is, the greater the reward, the greater the activation of the structures (Schultz et al., 2000; Zink et al., 2003). This is why (calorically-dense) ice cream or steak tastes better to us (on average) than a bowl of plain lettuce. In a situation of food scarcity, ingesting the food with more calories would keep us alive for a longer period of time. Drugs of abuse stimulate the reward pathway to a supra-biological extent – more than any natural reward would – and our brains interpret this stimulus as “life-giving”. The resources of attention, decision-making, memory and motivation are now focused on this ultimate reward. Is it any surprise, then, that the addict will pursue his/her drug of choice in spite of negative consequences?

The Endogenous Opioid System and Desensitization

This supra-physiological stimulation of reward centers and subsequent shift of cognitive resources comes at a significant price, however. Natural rewards that once brought pleasure (e.g., life or job
achievement/success, food, relationships) no longer produce the pleasure they once did. This process, known as desensitization in pharmacology, is defined as the gradual reduction in signal transduction following the activation of a receptor (Allouche et al., 2014). One mechanism underlying this phenomenon involves modifications in the opioid system.

The endogenous opioid system regulates numerous physiological functions including gastrointestinal transit, respiration, stress responses, immune functions, nociception, mood and hedonic sensations (Allouche et al., 2014; Le Merrer et al., 2009). The opioid receptor (OR) family is made up of mu (MOR), delta (DOR) and kappa (KOR) type receptors, all membrane receptors of the G-protein coupled receptor (GPCR) type. The endogenous ligands for the opioid receptor family are enkephalins, dynorphins and β-endorphin that are all derived from the proteolytic cleavage of larger precursor proteins: preproenkephalin, preprodynorphin, and proopiomelanocortin, respectively. Enkephalins, dynorphins and β-endorphin all contain an NH$_2$-terminal Tyr-Gly-Gly-Phe sequence that binds to opioid receptors (Le Merrer et al., 2009). Opioid receptors are generously expressed throughout the reward structures and a wealth of data implicates the intersection of opioid system and the reward pathway in the hedonic evaluation of food (Baldo and Kelley, 2007; Barbano and Cador, 2007; Olszewski and Levine, 2007), attachment (Moles et al., 2004; Young et al., 2001) and sexual reward (McKenna, 2002; Olszewski et al., 2007). Of note, morphine has the highest affinity for the MOR, and the majority of exogenous opiate effects are thought to be mediated through this receptor (Williams et al., 2013). The MOR is abundantly expressed in the caudate and putamen, and moderately expressed in the cortex, hippocampus, amygdala and spinal cord (Williams et al., 2013).

The link between OR activation and dopamine transmission is mediated through an indirect, disinhibiting mechanism. Dopaminergic neurons in the VTA receive both glutamatergic and GABAergic inputs from structures such as the amygdala, mPFC, NAc, and hippocampus. Within the VTA, MORs are primarily located on GABAergic interneurons. When activated, MORs,
which are coupled to the i/o class of G-proteins, inhibits these GABAergic neurons that primarily serve to inhibit DA neurons. Thus, MOR activation results in increased DA transmission in the VTA, and an increase in DA release throughout the reward pathway (Xi and Stein, 2002).

Desensitization within the opioid system largely involves phosphorylation of the ORs. Phosphorylation studies have found that agonist exposure brings about phosphorylation at several sites on ORs. Phosphorylation recruits β-arrestin that then both blocks the binding site for the G-protein (thus preventing activation of the receptor), and marks the receptor for internalization (Williams et al, 2013). Desensitization of the opioid system results in tolerance: the progressive need for more of a substance to produce the same effect. Although opioids are excellent in their therapeutic ability to mitigate pain, the opioid system appears to be uniquely sensitive to desensitization and tolerance. A study of patients chronically using opioids for pain management found nearly a ten-fold increase in opioid dose in little over a year (Buntin-Mushock et al, 2005).

**Mechanism of Action of Heroin**

The availability of heroin, like most substances, depends on the route of administration. The most common means of administration is intravenous injection. In this case, heroin by-passes first-pass metabolism and readily crosses the blood brain barrier due to the presence of acetyl groups which make it more fat-soluble than morphine. When it reaches the brain, it is deacetylated into the active metabolites 6-monoacetylmorphine (6-MAM) and morphine, and the inactive metabolite 3-monoacetylmorphine. 6-MAM and morphine both bind to MORs throughout the brain, cause disinhibition of DA neurons, and thus, bring about the characteristic effects of euphoria and pleasure. In the case of intravenous injection, heroin produces a feeling of intense pleasure, or rush, within the first minute, and this sensation lasts a few minutes. This is followed by approximately an hour of sedation. The analgesic effects last 3-5 hours. Smoking and snorting heroin are the next
most common routes of administration and also avoid first-pass metabolism of the liver (Habal, 2016). Oral administration is the least common route of administration, probably due to its blunted and gradual effect. When taken orally, heroin undergoes deacetylation during first-pass metabolism in the liver. This makes it a prodrug of morphine which is then delivered systemically (Sawynok, 1986).

While heroin is generally taken for the euphoric sensation it produces, it also has numerous other effects on the body. One of the most dangerous effects of opioid agonism is respiratory depression and irregularity. This effect is due to inhibition of several sites that regulate respiration including the pons and medulla (rhythm) and carotid and aortic bodies (chemoreceptors) (Pattinson, 2008). Opioids also produce bradycardia through inhibition of baroreceptors in the heart and hypotension because of a reduction in vascular resistance. Another common effect is itching, or pruritus, as opioids cause the release of histamine. Lastly, opioids are known to cause constipation due to an inhibition of gastric emptying and peristalsis (Habal, 2016).

The Addiction Cycle

Characteristics

Drug addiction is a disease of brain reward circuitry and chronic relapse characterized by 1) compulsion to seek and take drug, 2) inability to control drug-intake, and 3) the development of a negative emotional state when drug is not available. The spiral into addiction has been conceptualized to be driven by two processes representing alterations in brain systems: 1) the development of a hypo-responsive reward system, 2) sensitization to drug-related stimuli, and 3) the mounting recruitment of brain stress systems as an “opponent process” (Wise and Koob, 2014).
**Desensitization**

As discussed above, the reinforcing effects of drugs of abuse involves DA transmission from the VTA to the NAc. With repeated exposure to supra-physiological levels of DA, the reward system becomes progressively desensitized, and increasing amounts of drug are needed to produce the same pleasurable feeling (tolerance). Animals studies have found that cocaine, morphine and alcohol all produce increases in reward threshold (blunting of reward sensitivity) as measured through intracranial self-stimulation (Koob, 1996; Markou and Koob, 1991). An additional mechanism behind blunted reward circuitry may involve epigenetic mechanisms. For example, Cdk5 has been found to be upregulated in the NAc of mice with a history of chronic cocaine administration, and this upregulation is associated with histone H3 acetylation. Moreover, inhibition of Cdk5 in the NAc increases reward sensitivity (Wang et al, 2010).

**Sensitization**

A second emergent process has been labeled “sensitization” and involves the increased attention and an attribution of increased salience to drug cues and drug paraphernalia. This behavioral phenomenon is associated with dendritic arborization in the NAc and mPFC; an effect that may be mediated by upregulation of brain derived neurotrophic factor (BDNF), which promotes the survival and growth of new neurons and synapses (Kumar et al, 2005). In fact, injection of BDNF into the NAc increases the amount of work animals will perform to receive drug and it also increases relapse behavior (Graham et al, 2007).
Opponent Process

The opponent process theory of emotion/motivation proposed by Richard Solomon states that the emotion being experienced suppresses its opposite emotion (Solomon, 1980). In the case of addiction, it is used to characterize the emergence of a negative emotional and physical state and recruitment and sensitization of stress and pain systems that develops through repeated episodes of drug use and acute withdrawal. It is this second, negative affective aspect that is hypothesized to be the major motivator of continued drug use. As author and counselor Frank Tallis says, “At first, addiction is maintained by pleasure, but the intensity of this pleasure gradually diminishes and the addiction is then maintained by the avoidance of pain”. Indeed, drug addicts often remark that they no longer take drug to get “high” but instead to feel normal.

Individual Vulnerabilities to Addiction and Addiction-Models

Environmental Enrichment

In both human and animal studies, it has been well documented that the quality of one’s environment profoundly affects the addictive potential of a drug. As mentioned previously, investigation of heroin use amongst American soldiers in Vietnam revealed widespread heroin addiction. However, when these soldiers returned home, 95% discontinued heroin use (Robins et al, 2010). Improving the quality of the subject’s environment has been shown to mitigate addiction-like behavior in rodents as well. Environmental enrichment, in the pre-clinical setting, refers to the addition of physical and social stimuli to increase brain activity. For a rodent model, this could be the addition of a running wheel, cage-mates, smells and a variety of different objects to explore. Environmental enrichment has been found to have a positive effect on brain function: increasing
synaptogenesis (Diamond et al., 1964) and dendrite complexity (Greenough and Volkmar, 1973a; Greenough et al., 1973b), as well as cortical thickness (Diamond et al., 1966). Behaviorally, environmental enrichment may reduce, as well as serve as a treatment for, addiction-like behavior. Solinas and colleagues (Solinas et al., 2008) have shown that a 30-day environmental enrichment “treatment” can mitigate previously established conditioned place preference to cocaine. In contrast, negative environments or experiences can render a subject vulnerable to later drug-abuse, and early exposure to adverse life-experiences appears to be particularly detrimental. Childhood abuse and trauma have been associated with increased susceptibility to later alcohol, opiate and psychostimulant abuse (Khoury et al., 2010). Rats, that experienced maternal separation as pups, later go on to show greater acquisition of cocaine self-administration (Kosten et al., 2000).

**Passive Infusion vs. Self-Administration**

For many years, the molecular effects of various drugs of abuse have been studied by using experimenter-delivered models in which the animal receives injections (cocaine, amphetamine, heroin, etc.) or the inhalation of vapor (alcohol). However, this model of drug-abuse most likely lacks the complexity or even misrepresents drug-abuse in a human population that involves elements such as decision-making, learned associations, and self-administration behavior. Recent studies have demonstrated that even the molecular effects of a drug are different depending on whether that drug is received contingent (administered by the animal), or non-contingent (experimenter-delivered or yoked) upon behavior. For example, following self-administration (SA) of cocaine, VTA excitatory synapses are potentiated even after 3 months of abstinence; however, this effect is lost with experimenter-delivered cocaine (Chen et al., 2008). Moreover, non-contingent administration of drug has been shown to be stressful for the animal. Palamarchouk et al. (2009) showed that animals receiving yoked infusions of cocaine had far greater rises in mPFC
CORT than saline controls and animals that self-administered the drug. Previously, the Grigson laboratory demonstrated that animals receiving yoked administration of cocaine were more likely to avoid the cocaine-associated side of the chamber as well as avoid a saccharin cue that predicted the drug’s delivery (Twining et al, 2009a).

**Natural Reward Devaluation**

It is very likely that the most problematic side-effect of drug-addiction is the devaluation of natural rewards in the addicted person’s life. Things that once brought pleasure (e.g., social relationships and family, career fulfillment and attainment, etc.) now pale in comparison to seeking and attaining drug. This presents a significant problem for families and societies if the addicted individual now loses interest in caring for his/her children, loses interest in maintaining job security, or now sees family as a “bank” from which he/she can take money in order to acquire more drug. Reward devaluation can be modeled in the rodent by pairing the availability of a naturally rewarding sweet (saccharin) with the availability to self-administer a drug of abuse. In this model, approximately half of the animals will come to avoid the saccharin cue, and, in turn, show greater addiction-like behavior (i.e., shorter latency to self-administer, loading behavior, and drug intake (Grigson, 2008)). Moreover, suppression of intake of the drug-paired taste cue in the first few days of the study (at a time when drug intake is the same across animals) predicts the animal’s responsiveness to the drug as the study progresses. While there is much to be learned regarding the molecular mechanisms underlying this phenomenon, it may be a reflection of individual differences in sensitivity to reward salience, stress adaptability or greater adaptability of a system to preferentially seek out the most motivating reward at a given time.
The CRF System

CRF and the HPA Axis

Corticotrophin releasing factor (CRF) is a 41 amino acid neuropeptide discovered in the paraventricular nucleus (PVN) of the hypothalamus and characterized by Wylie Vale and colleagues in 1980 (Bale and Chen, 2012; Vale et al, 1981). It is most known for its role in activating the HPA axis, a leg of the autonomic nervous system. Stressful events cause the synthesis of the precursor protein, preproCRF which is cleaved into the mature protein (CRF) and released into the hypophyseal portal system from neurons in the hypothalamus. CRF then binds to CRFR1 receptors in the pituitary, causing the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the blood system. ACTH triggers the release of corticosterone (CORT) from the adrenal cortex (Costa et al, 1992). Circulating CORT then provides negative feedback on the HPA axis and further CRF production by binding to glucocorticoid receptors (GRs) in the central nervous system (Kretz et al, 1999).

The Extrahypothalamic CRF System

More recently, CRF is becoming recognized as integral to the orchestration of a more global central nervous system (CNS) stress response. Stress of various sorts causes the release of CRF in areas such as the amygdala (Roozendaal et al, 2002), locus coeruleus (Snyder et al, 2012) and the hippocampus (Chen et al, 2006). Further, both of its G-coupled receptors, CRFR1 and CRFR2 (Perrin and Vale, 1999) have several splice variants that are distributed differentially throughout the brain, resulting in a diverse and tissue-specific responses (Refojo et al, 2011).
CRF, the Hippocampus and Addiction

Within the hippocampus, the CRFR1s are the most plentiful, residing mainly on pyramidal cell bodies, dendrites, and within dendritic spines; a configuration indicative of an excitatory post-synaptic junction (Chen et al, 2004). Notably, these receptors are present mainly on pyramidal cells of the CA1 and CA3 region (i.e., the Schaeffer collaterals), the hippocampal region most associated with learning and memory (Chen et al, 2013; Chen et al, 2010). The endogenous ligand for the hippocampal CRFR1 appears to originate from basket cell interneurons that reside in the pyramidal layer and stratum radiatum (Chen et al, 2001). The axonal projections of these interneurons congregate around the CRFR1 on the pyramidal cells (Chen et al, 2000).

Like the effect of stress on the hippocampus originally attributed to the glucocorticoid receptor (Pavlides and McEwen, 1999), CRF concentrations in vitro and in vivo result in an inverted U shape in hippocampal function. Application of CRF to hippocampal slices facilitates LTP (Chen et al, 2001) and CRF injected directly into the brain enhances memory formation (Refojo et al, 2011). Indeed, it appears as though CRF-CRFR1 signaling is required for proper hippocampal function, as CRFR1 deficient mice demonstrate attenuated LTP (Schierloh et al, 2007), as well as learning and memory deficits (Contarino et al, 1999).

Conversely, chronic stress and application of CRF, at concentrations and durations that reflect a chronic stressed state, result in the retraction of dendritic spines (Chen et al, 2010; Ivy et al, 2010) and loss of LTP (Chen et al, 2013). Notably, the dendritic atrophy observed is not random but, instead, specifically targets thin dendritic spines, called “learning spines” (Bourne and Harris, 2008). These thin spines are thought to be the substrate of new memories, uniquely responsive to the patterned stimulation that promotes learning. With repeated stimulation, these thin spines begin to express AMPA receptors of the GLUR1 type and become the mushroom-shaped spines associated with memory consolidation (Bourne et al, 2008). This selective pruning of thin dendritic
spines observed in chronic stress and CRF application is mediated by RhoA, a GTPase that inhibits the polymerization of actin associated with synaptic plasticity (Chen et al, 2013).

**CRF, the mPFC and Addiction**

Among the many neuroadaptations associated with addiction, impairment in PFC- function is thought to be especially critical in facilitating drug-addiction. Both opiate and cocaine addiction are associated with impairments in memory, cognitive flexibility and decision making (Bechara, 2005); neuroadaptations that leave the addicted individual vulnerable to compulsive drug-use and relapse. Indeed, chronic drug use has been shown to lead to increased response to drug-related cues, and decreased control over drug-taking; behavior that is associated with increased glutamatergic transmission from the PFC to the NAc (Kalivas et al, 2005). In accordance with its role in attention and decision making, activation of the mPFC is necessary for cue and drug-induced reinstatement of heroin-seeking. Exposure to heroin-associated audio-visual cues results in cFOS expression in the mPFC, as well as heroin-seeking behavior (Koya et al, 2006). Moreover, inactivation of the dorsomedial PFC through the use of GABA_\text{A} and GABA_\text{B} agonists blocks cue-induced heroin-seeking (Rogers et al, 2008) and context-induced reinstatement of heroin seeking can be attenuated by pharmacologic inactivation of the ventral mPFC (Bossert et al, 2011). The mPFC is a region acutely vulnerable to the effects of stress on its ability to function. As a region rich in CRFR1, it should not be surprising, therefore, that excessive CRF signaling in the mPFC is associated with decreased cognitive flexibility, working memory and executive function. Moreover, acute periods of stress are associated with increases in CRFR1 mRNA expression (Uribe-Marino et al, 2016).
CRF, the Extended Amygdala and Addiction

The extended amygdala is proposed to play a pivotal role in drug-taking to combat the negative affect of withdrawal. Accordingly, naloxone-precipitated withdrawal is associated with increased excitability in this area (Gracy et al., 2001). Further, the stress neuropeptide, CRF, has been found to be elevated in the extended amygdala of dependent animals during episodes of withdrawal from drugs of abuse, including alcohol (Richter et al., 2000), nicotine (George et al., 2007), cocaine (Richter and Weiss, 1999), and opioids (Weiss and Koob, 2001).

CRF and Addiction

The “opponent process” or recruitment of pain and stress systems in drug addiction is thought to be mediated by the extrahypothalamic CRF system. As with other drugs of abuse, opioid dependence and withdrawal, in animals, is associated with stress-related symptoms such as: bodyweight loss and irritability (Navarro-Zaragoza et al., 2010), hypersensitivity to pain (Park et al., 2015), and conditioned place aversion (Lu et al., 2000). Notably, these symptoms can be attenuated through the administration of CRFR1 antagonists. Conditioned place aversion, the avoidance of an environment associated with an aversive state, is probably the most studied symptom of withdrawal in opiate addiction and can be readily elicited through the administration of an opioid antagonist such as naloxone to an opiate-dependent animal. Highlighting the role of CRF-CRFR1 signaling in this occurrence, naloxone-precipitated conditioned place aversion (CPA) is blocked through both systemic (Stinus et al., 2005) and intracerebral administration of CRFR1 antagonists (Heinrichs et al., 1995a). Moreover, CRFR1 knockout mice fail to develop CPA associated with opiate-withdrawal (Contarino and Papaleo, 2005). Finally, pretreatment with CRF
antagonists attenuates escalation of heroin self-administration in a long-access (8h and 12 h) model (Greenwell et al, 2009b; Park et al, 2015).

Addiction and Gene Expression

As explained in previous sections, the phenomenon of addiction is a vitiation or corruption of a system evolved to promote behaviors that enable survival of genes, organisms and species. Behavior, from a neuroscience perspective, can be explained in terms of neurons firing on, or influencing one another to bring about the ultimate goal of altering the movement of the body (e.g., through the pyramidal tracks and/or hypothalamus). Entrained behavior stems from well-worn “tracks” or strong and fast connections between relevant brain regions, and the strength of signals and connections depends on things such as physical connections between individual neurons and the responsiveness of neurons to a signal (see receptor desensitization). Whether we are following a pattern of behavior, or attempting to forge a new one, we are essentially strengthening the neural connections that bring about this behavior. Richard G. Scott, although not a neuroscientist, observed that “you become who you want to be by consistently being who you want to become”. External and internal factors cause the release of a symphony of neurotransmitters, moment by moment, and the activation of relevant receptors brings about a cascade of intercellular effects that alter the ability of the cell to receive future signals. One way this occurs is through gene expression, whereby information from a gene results in a gene-product (i.e., DNA is copied to RNA that is translated to protein). For example, strong depolarization of a cell often brings about the expression of structural proteins that will increase the “arborization” of the cell’s processes, thus increasing the likelihood of significant signal receipt in the future.

Drugs of abuse substantially alter the chemical communication between neurons and thus bring about significant structural cellular changes that set an animal or human up for becoming
dependent or addicted to the drug of abuse. It may not be surprising, then, that many have found changes in gene expression across the reward pathway in response to the ingestion of drugs of abuse. Kuntz-Melcavage and colleagues investigated gene expression in heroin self-administering rats after 1 day and 14 days abstinence, as well as in rats receiving non-contingent doses of heroin. In the mPFC, they observed an increase in the early response genes EGR1 and EGR2 in both the 1 day and 14 day abstinent rats. In the NAc, EGR2 was elevated in the 1 day rats and, surprisingly, dramatically increased in the non-contingent rats that had received 14 days abstinence (Kuntz et al., 2008a). A follow-up study utilizing DNA micro-array technology examined gene expression changes in the mPFC of the 14 day abstinent animals. The genes for BDNF, Calb1, Dusp5, Dusp6, EGR1, NPY, and Rgs2 were found to be differentially-expressed and associated with incubation of heroin-seeking (the phenomenon whereby abstinence following drug-self-administration increases behavioral responsiveness to the drug) (Kuntz-Melcavage et al., 2009).

Gene expression changes are not limited to opiate administration. Alcohol use has been associated with gene expression changes across the CNS in pathways involving GABA, glutamate, opioids, dopamine and serotonin as well as several other genes including BDNF and NPY (Worst and Vrana, 2005). Cocaine administration, as well, has been shown to produce changes in gene expression within the reward pathway. Using micro-array technologies, Freeman et al. (2010) examined gene expression differences in rats that underwent forced abstinence following cocaine self-administration for 1, 10 or 100 days. They found 1,461 genes differentially expressed in the mPFC and 414 genes in the NAc. Interestingly, some genes were changed acutely with cocaine SA (1 day animals), some genes changed with cocaine SA and remained changed into abstinence, and some genes only changed once the abstinent period began. Many genes in this study were largely involved in MAP kinase signaling, TNF signaling and synaptic plasticity (Freeman et al., 2010). In another study, Freeman et al. found genes such as EGR1, c-fos, NR4a1 and Arc to be
decreased in abstinence following cocaine SA, and these gene changes were associated with incubation of drug-seeking (Freeman et al, 2008).

**New Technologies for Gene Discovery**

The advent of new technologies has allowed researchers to effectively assess the status of a large number of genes in a time and cost effective manner. This is especially beneficial as most diseases are not caused or exacerbated by the mutation or expression of *one* gene, but rather a cluster of genes coupled with environmental factors.

**Microarray**

DNA microarrays allow researchers to examine the expression levels of a large number of genes in one experiment. A DNA microarray contains complimentary oligonucleotides (probes) of a set of chosen genes that will bind to the mRNA of those chosen genes within a sample. The target sequences are fluorescently labeled, and generate a signal once they are retained on the probe. The intensity of the signal, therefore, corresponds to the relative amount of mRNA of the given gene.

**RNA-Seq**

RNA sequencing (RNA-Seq) utilizes next generation sequencing (NGS) and allows researchers to assess the entire transcriptome of a given sample. Moreover, it has the added benefit of *absolute* quantitation, rather than *relative* quantitation of microarray and qRT-PCR, as the reads of a given gene are counted rather than relying on the strength of a signal.
In brief, cDNA is reverse transcribed from isolated RNA. The cDNA is then fragmented and oligonucleotides containing complimentary adapter sequences and barcodes are ligated to the fragmented cDNA. One part of the adapter allows the cDNA to attach to the flow cell during amplification, while the barcode portion of the adapter (unique to each sample) allows the samples to be sorted in the following analysis. The cDNA library is then sequenced and read counts are generated. The reads are then aligned to the species-appropriate genome, normalized and analyzed for differential mRNA expression.

**BiSulfite Amplicon Sequencing**

BiSulfite Amplicon Sequencing (BSAS) is another technique that utilizes NGS. BSAS is used to assess cytosine methylation in a discrete portion of a chosen gene. First, genomic DNA is incubated with sodium bisulfite. This step deaminates cytosines and converts them to uracils, *unless* the cytosine has a methyl group at the 5’ position, in which case, it is protected from conversion and remains a cytosine. Primers designed to target short (250-400bp) regions of DNA and the bisulfite converted DNA is then amplified by PCR. During amplification, uracils are converted to thymines. The resulting amplicon is then made into a library and sequenced and aligned to the reference genome. Percent methylation at a particular site is calculated by the proportion of converted and unconverted cytosines (C/C+T) (Masser *et al*, 2015)

**Conclusions**

Drugs of abuse exert their effects through excessive stimulation of the reward pathway, thus tricking the brain into responding to the drug as though it is an exceptionally life-giving reward. Moreover, continued drug use brings about neural adaptations such as desensitization (the...
increasing need for more drug to produce the same effects), sensitization (tuning-up of the reward pathway to preferentially respond to drug-associated stimuli) and the emergence of a negative physical and emotional state in the absence of drug. These multifaceted effects conspire to make drug-addiction an intractable condition. As drugs of abuse have a profound effect on changing brain chemistry, synaptic plasticity, gene-expression, and ultimately, behavior, it is important for researchers to investigate these drug-induced alterations in order to develop effective treatment strategies.
Chapter 2

Reward Devaluation and Heroin Escalation is Associated with Differential Expression of CRF Signaling Genes


Abstract

One of the most damaging aspects of drug addiction is the degree to which natural rewards (e.g., family, friends, and employment) are devalued in favor of seeking, obtaining and taking drugs. We have utilized an animal model of reward devaluation and heroin self-administration to explore the role of the CRF pathway. When given access to a saccharin cue followed by the opportunity to self-administer heroin, animals will parse into distinct phenotypes that suppress their saccharin intake and then escalate heroin self-administration or vice versa. We find that large saccharin suppressors (large heroin takers, LS) demonstrate increased mRNA expression for elements of the CRF signaling pathway (CRF, CRF receptors and CRF binding protein) within the hippocampus, medial prefrontal cortex and the ventral

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1 This Chapter is comprised of material from a published manuscript [McFalls et al., Reward devaluation and heroin escalation is associated with differential expression of CRF signaling genes. Brain Res. Bull. 2016:1229-1235.] that has been supplemented with independent, but complementary, studies involving pharmacological interventions. The initial behavioral self-administration of heroin experiment was designed and executed by CGI (in consultation with PSG). All the molecular experiments were designed and executed by AJM (in consultation with WMF and KEV). GB assisted with RNA extraction and provided training on RT-PCR. AJM prepared the manuscript and all Figures (with editorial assistance from PSG, WMF, and KEV). The data in Figure 2-2 were collected by CGI. The data in Figures 2-8 and 2-9 were collected by DA. All remaining data were collected by AJM.
tegmental area. Moreover, there were no gene expression changes of these components in the NAc. Use of bisulfite conversion sequencing suggests that changes in CRF binding protein and CRF receptor gene expression may be mediated by differential promoter methylation. To explore the hypothesis that CRF signaling was contributing to drug-abuse liability, we employed the use of a potent and selective CRFR1 antagonist, Antalarmin, on an independent set of animals. Twelve male rats were subjected to the saccharin-heroin pairing for 16 trials, at which point they were split into LS and Small suppressors (SS) according to saccharin intake on trial 16. These animals then went through 2 weeks abstinence from drug and then were placed back into the self-administration chamber to assess heroin-seeking (extinction/reinstatement, E/R). 1 hour prior to E/R, half of the LS and half of the SS were given an IP injection of Antalarmin. Antalarmin significantly reduced heroin-seeking across groups and was most effective in the LS group, indicating that CRF signaling may mediate drug-seeking behavior.
Introduction

The Natural Reward Pathway

Evolution has developed a system to enable the survival of organisms and their genes, whereby attention and memory are allocated to the most salient experiences, whether good or bad. The enjoyment of natural rewards (e.g., highly caloric food, sex, social relationships) activates a reward pathway involving a dopaminergic projection from the VTA to the NAc and prefrontal cortex (PFC) (Kelley and Berridge, 2002; Wise, 1996; Wise and Rompre, 1989). Conversely, threatening stimuli elicit activation of fight or flight pathways, the autonomic nervous system and the hypothalamic-pituitary-adrenal axis (HPA axis) (Goldstein, 2010). Activation of either or both of these systems actively engages these brain regions and is associated with synaptic plasticity (Joels and Baram, 2009; Kim and Diamond, 2002). Drugs of abuse hijack these neural reward substrates in a maladaptive fashion (i.e., greater intensity of reward for drug-taking and stressful responses to drug withdrawal). Drug abuse results in physiological responses such as dysregulated stress responses (Koob and Kreek, 2007), excessive attention to drug cues (Robinson and Berridge, 2008), cue-induced withdrawal (Koob and Le Moal, 2001), and the devaluation of natural rewards; as exemplified by drug users’ propensity to be underweight (McIlwraith et al, 2014) and to feel socially isolated (Nair et al, 1997). Arguably, then, it is not the consumption of drug that is the most pervasive and costly problem of drug abuse, but the degree to which other things of life pale in comparison to seeking and acquiring drug.

The Devaluation of Natural Reward/Reward Comparison Model

Regardless of the addictive potential of a drug, there remain vast individual differences in addiction liability of the user. For example, it is approximated that half of first time users of heroin transition
to dependence (SAMHSA, 2014). Therefore, it is imperative to discover the underlying mechanisms of vulnerability and resilience to drug-addiction.

We have developed a rodent model of heroin self-administration (SA) and reward devaluation that models the variability seen in human drug addiction. Rats that most forgo a drug-paired taste cue exhibit the greatest responding for drug (i.e., a shorter latency to self-administer, greater loading behavior, and greater drug intake (Grigson, 2008)). Further, suppression of intake of the drug-paired taste cue early in the study (at a time when drug intake between populations is indistinguishable) predicts future drug use. Much like a human population, we observe striking individual differences in behavioral responses to different rewards in this animal model; approximately half of the animals in this paradigm avoid the drug-paired saccharin cue and these rats demonstrate high responding for heroin (Imperio and Grigson, 2015). The mechanisms that underlie this phenomenon are unknown; however, it may be a reflection of individual differences in perceived reward salience or greater adaptability of a system to preferentially seek out the most motivating reward at a given time.

**Molecular Mechanisms of Individual Preferences for Drugs of Abuse**

In addition to neuroadaptation caused by drug exposure, recent studies have begun to elucidate underlying mechanisms of individual preference for various drugs of abuse. For example, a transcriptome study of the rat nucleus accumbens revealed distinct gene expression profiles between subjects that demonstrated high or low preference for ethanol (Bell et al., 2009). Further, Zhou et al. (2015) demonstrated that individual differences in responding to heroin-primed reinstatement were linked with differences in gene expression for vasopressin, D2 receptor, and orexin (Zhou et al., 2015). Our group has previously shown that avoidance of an otherwise palatable saccharin cue following pairings with cocaine or morphine is associated with real time changes in
neurotransmitter and hormone levels, such as blunted accumbal dopamine levels and increased corticosterone response to presentation of the drug-paired cue, as well as increases in withdrawal symptoms (Gomez et al., 2000; Grigson and Hajnal, 2007; Nyland and Grigson, 2013). Imperio et al. (2016) also show differences in gene expression in the NAc between rats that greatly avoid a saccharin cue paired with the opportunity to self-administer heroin (referred to as the large suppressors) and those that do not (referred to as the small suppressors) (Imperio et al., 2016). As the saccharin component of our model not only serves as a comparator, but also as a cue for the coming drug, we proposed that the reward-devaluation we observe has a strong memory and attentional/decision-making component. Given the known role of corticotropin releasing factor (CRF) signaling in the CNS stress-response, learning and memory, cognitive flexibility and drug-taking behavior, we investigated the expression of genes related to these phenomena (Figure 2-1) in key brain regions for attention, learning and reward: the mPFC, hippocampus, VTA and NAc. Of note, the International Union of Basic and Clinical Pharmacology recommends that the peptide (CRF) and its receptors (CRFR1 and CRF2) be referred to by the term “corticotropin releasing factor” as opposed to the frequently used CRH (corticotropin releasing hormone). Nevertheless, the gene is still denoted as corticotropin releasing hormone (CRH) (http://www.ncbi.nlm.nih.gov/gene/81648).

Epigenetic regulation has the potential to underpin the long-lasting ‘memory’ of addiction that persists even with drug abstinence (Nestler, 2014). As long-lived post-mitotic cells, neurons have the capacity to carry long-lasting epigenetic changes. Previously, we have examined chromatin status with stimulant abuse (Freeman et al., 2008) and recent reports provide compelling evidence for epigenetic changes with opiates (e.g., Koo et al., 2015). Both histone and DNA modifications (cytosine methylation) are key epigenetic regulators of genomic structure and gene expression. CpG methylation refers to the addition of a methyl group (CH₃) to the #5 position of the nucleotide cytosine when it is followed by the base guanine. Of note, although the genome is
largely depleted of CpGs, CpG-rich areas can be found in gene regulatory areas such as the transcription start site (TSS) and the gene body (Jones, 2012). Methylated cytosines (5mC) in genomic promoter regions generally repress gene expression by recruiting methyl-CpG binding domain proteins that then attract repressor complexes, resulting in a condensed chromatin structure (Jones, 2012; Jones et al, 1998). The present studies focus on gene expression and DNA methylation. This is of particular relevance to drug abuse as 5mC can be both a persistent molecular signal lasting the lifetime of a cell, even being passed on to daughter cells (Holliday, 2006), or be dynamic, with *de novo* methylation followed by de-methylation occurring in a matter of minutes (Day and Sweatt, 2010). Therefore, a second aim of this study was to examine whether genes that demonstrated differential mRNA expression were associated with differential methylation status of their promoter regions.
Figure 2-1. – Hypothetical hippocampal pyramidal neuron illustrating the molecular targets that are the subject of this study.
Materials and Methods

Behavioral Analysis

Subjects and Surgeries

Outbred, male Sprague-Dawley rats \((n=20)\) were obtained from Charles River. Rats were implanted with an intrajugular catheter and were given one week to recover as previously described (Twining et al, 2009a). These animals also contributed to an independent NAc RNA-Seq study (Imperio et al, 2016). In order to investigate the molecular mechanisms of individual differences brought about through this behavioral paradigm, animals that demonstrated the most robust behavioral effects were chosen for the experiments outlined herein \((n=8, \text{see } \text{“Selection of Large and Small Suppressors”})\). For detailed behavioral experiments and data on the complete set of animals, see Imperio & Grigson, (2015) (Imperio et al, 2015). Within this Chapter, these animals are referred to as “experiment 1”.

In a second study involving the use of pharmacological intervention, 12 outbred male Sprague-Dawley rats were subjected to the same protocols as the first study. That is, they were implanted with an intrajugular catheter, given one week to recover, and were subject to the saccharin-heroin pairing (see behavioral testing). These animals are referred to as “experiment 2”.

Apparatus

Habituation and SA experiments were carried out as described previously (Twining et al, 2009a). Each chamber was equipped with a light over the “active spout”, a tone generator (2900 Hz), and three retractable spouts. The leftmost spout delivered saccharin, the middle spout served as the
“inactive” spout and the rightmost spout served as the “active” spout. Licks on each spout were recorded by a lickometer circuit (for further details, see (Imperio et al, 2016) and (Imperio et al, 2015)).

Behavioral Testing

Habituation: In order to provide familiarity to the self-administration chambers and to establish the behavioral task (spout licking), all animals initially underwent three days of habituation to the self-administration chambers, as we have described previously (Twining et al, 2009a). During habituation, the rats were mildly water restricted (25 mL/day). On the first day, water was available from the leftmost spout, the second day from the future “inactive” middle spout and on the third day, water was available from the future “active” spout. Thereafter, the rats were returned to ad libitum access to food and water.

Acquisition: During acquisition, the rats were placed in the self-administration chamber where, for the first 5 minutes, they received access to saccharin through the left-most spout. After 5 minutes, the first spout retracted and two spouts, active and inactive, extended into the chamber. The rats were then given 6 hours access to heroin on an FR10 schedule of reinforcement where ten licks on the active empty spout resulted in a 6 second infusion of either saline (n=6) or 0.06mg/0.2ml heroin (n=14)). Upon completion of the infusion, the spouts retracted and the house light came on, which signaled an additional 14 second time-out period (i.e., additional responses on the active spout were not rewarded for a total of 20 sec). Rats were given 6 hours to self-administer saline or heroin 5 days/week for a total of 16 trials. Catheters were flushed with heparinized saline before and after each SA session. Patency was verified, when indicated, using an IV infusion of propofol.

Escalation: Escalation of drug use is defined as the gradually acquired loss of control over drug consumption (Ahmed et al, 2000). Escalation of heroin self-administration is assessed by
examining the number of infusions of heroin taken during the 1st hour of access across acquisition trials 1 – 16 (Figure 2C).

**Progressive Ratio Challenge (PR):** The PR challenge determines how hard the rat is willing to work for a drug infusion. After the acquisition phase, each rat was presented with a single PR challenge to test their motivation to work for drug. During the PR challenge, subjects were initially presented with the 5 min saccharin cue. After 5 min access to the saccharin cue elapsed, each subject started on an FR 10 on the active spout for the 1st infusion, with subsequent infusions requiring an increasing number of licks to earn the next infusion: 10, 12, 16, 22, 30, 40, 52, 66, 82, 100, 120, 142, 166. The session was terminated when 30 min elapsed without a rat having earned a reinforcer. After the PR challenge, the rats were returned to the FR10 schedule of reinforcement for three maintenance trials.

**Extinction/Reinstatement (E/R):** Extinction and reinstatement assess persistence of drug-seeking. During the extinction trial, the rat was placed in the SA chamber and given 5 minutes access to the saccharin solution as before. After this, the rat underwent a 6 hour extinction session in which no amount of licking of active or inactive spouts was rewarded with drug infusion. Otherwise, all test conditions were as described (see acquisition). At the end of the extinction session, rats were given a single non-contingent IV infusion of heroin (0.06 mg/0.2ml) and licks on the active and inactive spouts were recorded for 1 additional hour in which responding produced no infusion of heroin.

**Abstinence:** In experiment 2, the animals were subject to a two week abstinence period during which they remained in the home cage and were not given access to saccharin or heroin. This is to model a forced abstinence period in humans whereby a drug-addicted person goes to a drug-rehabilitation facility, or other place in which he or she has no access to drugs.
**Administration of a CRFR1 Antagonist**

In experiment 2 (see *subjects and surgeries*), we tested the hypothesis that a CRFR1 antagonist that crosses the blood brain barrier would disrupt drug-seeking behavior in the E/R behavioral testing experiment. Twelve Sprague-Dawley rats were subject to the saccharin-heroin SA paradigm as before and grouped as LS (n=5) and SS (n=6) based upon our previously established criteria of below (LS) and above (SS) 200 licks of saccharin in the 5 minute saccharin availability on the final day of acquisition (day 16) (Figure 2.7) (Imperio *et al*, 2015). Of note, one subject was eliminated from the study as he showed no interest in either saccharin or heroin throughout the acquisition period. Following the abstinence period, we assessed the effect of a CRFR1 antagonists on drug seeking behavior. One hour prior to the E/R test, 3 rats in the LS group and 3 rats in the SS group were given an IP injection of Antalarmin, a selective and potent antagonist of the CRFR1 receptor, whereas, 2 rats in the LS group and 3 rats in the SS group were given an IP injection of vehicle. The rats were again put into the self-administration chamber where they were given an E/R test (Figure 2.8). Antalarmin was suspended (via sonication) in carboxymethyl cellulose (5 mg/mL) dissolved in PBS (pH 5.5) and delivered as 10 mg/kg. Vehicle was carboxymethylcellulose (5 mg/mL) dissolved in PBS (pH 5.5) (Zorrilla *et al*, 2002).

**Sacrifice and Dissection**

All animals were sacrificed by live decapitation 24 hours after the last E/R session. Dissection was conducted as described previously (Vanguilder and Freeman, 2011) on animals from experiment 1. Harvested brain regions included Hippocampus, NAc, VTA and Medial Prefrontal Cortex. Tissues were flash frozen and stored at -80°C until they were processed for molecular analysis.
Molecular Analysis

*Gene Expression Analysis*

Genes analyzed for differential expression have been previously associated with CRF signaling and drug SA, learning and memory, and stress (Deroche-Gamonet *et al.*, 2003; Koob *et al.*, 2001; Meaney *et al.*, 2002; Nunez *et al.*, 2007) (Table 1). RNA for gene expression analysis and DNA for bisulfite conversion sequencing was isolated using the Allprep DNA/RNA kit (Qiagen). Complementary DNA (cDNA) was generated from the isolated RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Messenger RNA expression of CRH and associated genes (Table 1) were assessed though quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The $2^{-\Delta\Delta CT}$ method was employed to assess relative gene quantification with beta actin as the endogenous control (VanGuilder *et al.*, 2008). TaqMan gene expression assays were obtained from Life Technologies.

*Bisulfite Conversion Sequencing*

Bisulfite conversion sequencing was performed on DNA obtained from animals and tissues from the behavioral experiments. Briefly, isolated genomic DNA was bisulfite-converted using the EZ DNA Methylation Lightning kit (Zymo Research). Primers were designed to amplify CpG islands in the proximal promoter of target genes where CpG islands were present (Table 2). Notably, not all genes contain CpG islands, and may rely on other means for transcriptional regulation (Deaton and Bird, 2011). Regions of interest were amplified using bisulfite-converted DNA template, forward and reverse primers and ZymoTaq DNA polymerase. Prior to sequencing, amplified DNA was resolved on a DNA gel containing ethidium bromide and imaged under UV light to confirm
correct amplicon size. Next, amplicons were purified using the QIAquick gel extraction kit (Qiagen). Purified amplicons were sequenced using bisulfite converted sequencing primers. In order to capture the entire sequence, internal primers were also used in sequencing reactions (Table 2).

**Selection of Large and Small Suppressors**

In order to explore the basis of the divergent behavioral phenotypes, the top 4 large suppressors (LS) and the bottom 4 small suppressors (SS) were chosen from a larger set of animals (see (Imperio et al, 2015) for behavioral data of the full set of animals) for molecular analysis (experiment 1). In the original set of animals, LS and SS classification was based on saccharin licks on day 16 of acquisition (i.e. SS >200 licks, LS < 200 licks). The selection of animals from this group for our molecular analysis was based on differences in saccharin intake between the first two days and the last two days of the 16 day acquisition period when the behavior of high drug-taking and lower drug-taking rats was clearly distinguishable. This enabled us to use the animals with the greatest differences in behavior as exemplars to explore the molecular basis of the different phenotypes.

In experiment 2, LS and SS were defined based upon saccharin suppression on day 16 of the saccharin-heroin pairing. As before, LS were those rats who had less than 200 licks on the saccharin spout, SS were rats who had more than 200 licks on the saccharin spout (see (Imperio et al, 2015)). For selection of animals who received Antalarmin or vehicle, see Administration of a CRFR1 Antagonist.
Statistics

Gene expression was normalized to the saline group (n=7) and standard t-tests were used to compare differences between LS and SS. Of note, aside from normalization, saline animals were not included in the analysis; the goal of the study was to uncover differences that underpin heroin-induced suppression of saccharin intake, and the saline animals had not been typed by the model (i.e., presumably, the saline animals are a mix of LS and SS). We chose to normalize our qRT-PCR data to the saline animals because they represent a mixed population that has not been exposed to drug. Utilizing the saline controls as a normalization standard permits a comparison of different genes because they are scaled to a common starting point. For example, if we normalize to one phenotype, we will not know whether a gene has higher or lower expression. Using this approach, we can address whether a gene expression phenotype is increased or decreased relative to a mixed, outbred Sprague-Dawley population. Percent methylation of each targeted and amplified CpG site was determined by measuring the peak height of unconverted Cs divided by the total peak height values for that site (C/[C+T]) (Wang et al, 2013). A standard t-test determined significance between groups (p<0.05).

In experiment 2, the difference between the effect of Antalarmin or vehicle on extinction behavior in hour 1 was determined by a standard t-test (Figure 2-8A). Then, a two-way ANOVA was employed to determine whether there was an effect of group or treatment between the 4 experimental groups in the first hour of extinction. Two-way ANOVAs were performed on hours 2-6, as well (data not shown). Again, a standard t-test was used to determine the effect of Antalarmin or vehicle on reinstatement behavior, then a two-way ANOVA was employed to explore the effect of treatment or group on reinstatement.
Results

Drug Self-Administration and Saccharin Suppression

In experiment 1, the subset of 4 rats/group, the LS rats exhibited greater avoidance of the heroin-paired saccharin cue than did the SS and this greater avoidance of the saccharin cue was associated with greater heroin self-administration (see Figure 2-2). This subset of rats, like the larger population (Imperio et al, 2015) also exhibited differences in escalation of 1st hour heroin self-administration across trials. Thus, LS rats escalated 1st hour heroin self-administration from trial 1 – trial 16, while the rats in the SS condition did not (see Figure 2-2, panel C). For further behavioral analyses related to the larger data set, see (Imperio et al, 2015). Subjects selected for this study were from the second replication in Experiment 2 outlined in (Imperio et al, 2015).

In experiment 2 described here, LS and SS demonstrated differential saccharin suppression on trials 9-16. Heroin intake differed between LS and SS on trials 4, 5, and 13-16.

Saccharin Suppressions is Associated with Differential Expression of CRF Signaling Molecules

Hippocampus

Within the hippocampus, we found a diminished expression of CRHR1 mRNA in the small suppressor group compared to the large suppressor group. Similarly, the expression of CRHR2 mRNA was higher in the large suppressor group than the small suppressors (Figure 2-3). Moreover, while they did not achieve statistical significance, the mRNAs for both CRH and its regulatory binding partner (CRH binding protein (CRHbp)) were lower in the small saccharin suppressors (small heroin takers).
**Medial Prefrontal Cortex (mPFC)**

Similar to the hippocampus, small suppressors demonstrated lower expression levels of the CRHR1 gene (Figure 2-4). Once again, mRNA levels for both CRH and CRHbp were higher in the large suppressors (but not statistically different from the small suppressors). Additionally, large saccharin suppressors demonstrated increased mRNA expression for GLUR1.

**Ventral Tegmental Area (VTA)**

Within the VTA, we observed increased expression of the mRNAs for CRFbp and Cdk5 in the large suppressor group, while the mRNA expression for β-Catenin and Rgs4 was reduced in the large suppressors (Figure 2-5).

**Nucleus Accumbens (NAc)**

No differential expression of targeted genes was observed between our two groups in the NAc (Figure 2-6).

**Correlational Analysis between Gene-Expression and Behavior**

Of note, we performed correlative analysis of mRNA expression with saccharin suppression and heroin intake and while there was a trend, nothing was statistically significant. As speculation, this may indicate that our genes interact to bring about behavioral phenomena, or that our findings are merely one aspect of the emergence of individual differences in drug-taking behavior (data not shown). Additionally, it should be noted that the small sample size (n=4) herein may preclude the observance of gene-behavior interactions.
**Bisulfite conversion sequencing**

**CRH Binding Protein**

Within the VTA, we examined the methylation levels of 12 CpGs in the proximal promoter region of the CRHbp gene (i.e., -158 to +168 relative to the transcription start site; Figure 2-7). Although sequence anomalies precluded accurate measurement of 4 CpGs, we found one CpG (CpG 4) to have significantly less methylation in the large suppressor’s compared to the small suppressors. Further, the difference in methylation levels of the adjacent CpG (CpG 5) approached significance (p=0.07). Moreover, with the exception of CpG 9, all other CpGs trended toward less methylation in the large suppressor group.

**CRHR1**

We also examined 59 CpGs in the proximal promoter region of the CRHR1 gene in the hippocampus. All except one (-208 relative to the transcription start site) were found to be completely un-methylated. The site that exhibited methylation was not statistically different between large suppressors and small suppressors, although there was a small trend toward less methylation in the large suppressors at this CpG (data not shown).

**Antalarmin**

The results of the t-tests revealed an overall difference in extinction-responding in hour 1 between animals who received vehicle or Antalarmin (p<0.01) (Figure 2-9A). Moreover, the 2-way ANOVA revealed an effect of treatment between both LS and SS (p=0.006) (Figure 2-9A&B). The
two-way ANOVA was conducted for hours 2-6 of the extinction session and no difference between groups was detected. No difference was found between vehicle and Analarmin groups in both t-test (vehicle vs Antalarmin) and 2-way ANOVA (4 treatment groups) in the reinstatement test (Figure 2-10).
Figure 2-2. – Saccharin suppression is associated with heroin intake.

Rats that increase their intake of saccharin over sequential trials do not increase their intake of heroin, whereas rats that suppress intake of saccharin increase heroin intake. Panel A represents saccharin intake and heroin intake of selected Small Suppressors and Large Suppressors over 16 saccharin + heroin pairings. Panel B represents the change in saccharin and heroin intake on days 15 and 16 in the small and large suppressor groups when compared to day 1 (day 1 = 100%). Panel C represents heroin escalation as measured by heroin infusions in hour 1 of the acquisition sessions. Small Suppressor and Large Suppressors used in this study were chosen from a larger set of rats whose behavioral data is published in (Imperio et al, 2015). Small suppressors (SS) in black, Large Suppressors (LS) in red, (SS, n = 4; LS, n = 4)
Figure 2-3. – Saccharin suppression is associated with differential expression of CRF associated molecules in the Hippocampus.
Small suppressors (SS) in black, Large Suppressors (LS) in white (SS, n = 4; LS, n = 4)
Figure 2-4. – Saccharin suppression is associated with differential expression of CRF associated molecules in the mPFC.

Small suppressors (SS) in black, Large Suppressors (LS) in white (SS, n = 4; LS, n = 4)
Figure 2-5. – Saccharin suppression is associated with differential expression of CRF associated molecules in the VTA.

Small suppressors (SS) in black, Large Suppressors (LS) in white (SS, n = 4; LS, n = 3)
Figure 2-6. – Saccharin suppression is associated with differential expression of CRF associated molecules in the NAc.

Small suppressors (SS) in black, Large Suppressors (LS) in white (SS, n = 4; LS, n = 4)
Table 2-1. Genes selected for mRNA expression analysis.

As described in Figure 2-1, the selected genes are involved in CRF signaling and exhibit differential gene expression in distinct brain regions (Figures 2-3, 2-4, 2-5, 2-6).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>RT-PCR Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
<td>Rn01462137_m1</td>
</tr>
<tr>
<td>CRHbp</td>
<td>CRH binding protein</td>
<td>Rn00594854_m1</td>
</tr>
<tr>
<td>CRHR1</td>
<td>CRH receptor type 1</td>
<td>Rn00578611_m1</td>
</tr>
<tr>
<td>CRHR2</td>
<td>CRH receptor type 2</td>
<td>Rn00575617_m1</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Glucocorticoid receptor (GR)</td>
<td>Rn00561369_m1</td>
</tr>
<tr>
<td>HspB1</td>
<td>Heat-shock protein beta 1</td>
<td>Rn00583001_g1</td>
</tr>
<tr>
<td>GRIA1</td>
<td>Ionotrophic Glutamate receptor 1</td>
<td>Rn00709588_m1</td>
</tr>
<tr>
<td>Rgs4</td>
<td>Regulator of G protein signaling</td>
<td>Rn01490867_g1</td>
</tr>
<tr>
<td>Fgfr2</td>
<td>Beta-Catenin</td>
<td>Rn01269940_m1</td>
</tr>
<tr>
<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
<td>Rn04219635_m1</td>
</tr>
</tbody>
</table>
Figure 2-7. – Differences in CRFbp mRNA expression is associated with differential methylation status of CpGs in the proximal promoter of the CRHbp gene.

A) Gene map of CRFbp gene, primer placement and sequencing results. B) Percent 5-mC of the targeted CpGs. NM: Not measured - Sequencing anomalies prevented measurement of this CpG. C) Magnified view of the sequence of CpGs 4 and 5. Small suppressor exemplar on top, large suppressor on bottom.
Figure 2-8. – Saccharin suppression and heroin intake in behavioral experiment 2.

A. Approximately half of the rats suppress saccharin intake when it is paired with the availability to self-administer heroin. B. Heroin intake differs between LS and SS. This set of animals was later subjected to pharmacological intervention with Antalarmin, a CRFRI antagonist (see Figure 2.8). * denotes p<0.05, ** denotes p<0.01, *** denotes p<0.001
Figure 2-9. – Antalarmin disrupts heroin-seeking behavior.

A. Six hour extinction testing after 1 hour pretreatment with Antalarmin or vehicle.  B. Extinction behavior among LS and SS pretreated with Antalarmin or vehicle.  C. Two-way ANOVA revealed an effect of drug on heroin seeking in hour 1 of extinction session. ** denotes p<0.01
Figure 2-10. – Reinstatement behavior among LS and SS following extinction and pre-treatment with Antalarmin or vehicle.

Following extinction, all animals were given 1 non-contingent infusion of heroin (0.06mg/0.2ml) to reinstate drug-seeking behavior. Each infusion attempt represents 10 licks on the active spout. A. T-tests revealed no differences between infusion attempts of the Antalarmin group (n=6) and the vehicle group (n=5). B. The two-way ANOVA revealed no differences in infusion attempts between the 4 experimental groups. LS-VEH: large suppressor receiving vehicle (n=3), LS-ANT: large suppressor receiving Antalarmin (n=2), SS-VEH: small suppressor receiving vehicle (n=3), SS-ANT: small suppressor receiving Antalarmin (n=3).
Discussion

These experiments highlight the potential dynamic regulation of CRF signaling in response to heroin reward. In the present study, differential expression of mRNAs for CRF signaling were observed throughout the memory and reward pathways. Not only do drugs of abuse frequently cause desensitization of the reward pathway (thereby requiring more drug to elicit rewarding effects), they also bring about recruitment and sensitization of stress (Koob, 2009b) and pain systems (Edwards et al., 2012), through repeated episodes of acute withdrawal. It is this second, negative affective aspect that is hypothesized to be the major motivator of continued drug use, and is thought to be mediated, in part, by the extrahypothalamic CRF system (Koob, 2010). As with other drugs of abuse, opioid dependence and withdrawal, in animals, is associated with stress-related symptoms such as: bodyweight loss and irritability (Navarro-Zaragoza et al., 2010), hypersensitivity to pain (Park et al., 2015), and conditioned place aversion (Lu et al., 2000). Notably, these symptoms can be attenuated through the administration of CRFR1 antagonists (Lu et al., 2000; Navarro-Zaragoza et al., 2010; Park et al., 2015). Further highlighting the role of CRF-CRFR1 signaling in this process, naloxone-precipitated conditioned place aversion (CPA) is blocked through both systemic (Stinus et al., 2005) and intracerebral administration of a CRFR1 antagonist (Heinrichs et al., 1995b). Moreover, CRFR1 knockout mice fail to develop CPA associated with opiate withdrawal (Contarino et al., 2005). Finally, pretreatment with CRF antagonists attenuates escalation of heroin self-administration in a long-access (8h and 12h), but not short access (1 h) animal self-administration model (Greenwell et al., 2009a; Park et al., 2013).
Extrahypothalamic CRF System and Hippocampus mPFC circuit

Corticotropin Releasing Factor (CRF) is a 41 amino acid neuropeptide most known for its role in activating the HPA axis; that is, causing the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary to trigger the release of corticosterone (CORT) from the adrenal cortex in rodents (Costa et al, 1992). More recently, CRF is becoming recognized as integral to the orchestration of a more global central nervous system stress response. Stress of various sorts causes the release of CRF in areas such as the amygdala (Roozendaal et al, 2002), locus coeruleus (Snyder et al, 2012) and the hippocampus (Chen et al, 2006). Further, both of its G-coupled receptors, CRFR1 and CRFR2 (Perrin et al, 1999), have several splice variants that are distributed differentially throughout the brain, resulting in a diverse and tissue-specific response (Refojo et al, 2011).

While not all changes reached statistical significance within this set of 4 subjects/group, the common pattern between the expression of CRH, CRH receptors and binding protein mRNA in the mPFC and hippocampus is striking. This is particularly so given the strong facilitatory role stress plays in attention and memory and the associative component of our self-administration model. Based on mRNA changes (and assuming they reflect protein levels), the sum total of the responses predict increased CRF tone. This increased tone could serve to strengthen associative learning and underlie reward devaluation and escalating heroin self-administration. The hippocampus and mPFC are connected in both direct and indirect ways, a circuit that is integral to associative learning (Li et al, 2015). Notably, hippocampal connections to the mPFC originate from the CA1 region where CRF and CRF receptors are highly expressed. Further, neurons of the hippocampus have been found to fire synchronous theta rhythms with a subset of neurons in the mPFC during learning, goal-directed behavior and REM sleep (an important component of memory consolidation) (Hyman et al, 2005; Li et al, 2015). The effects of stress and CRF on attention and
memory have a bidirectional effect; for example, acute application of CRF to hippocampal slices facilitates LTP (Chen et al., 2001) and CRF injected directly into the brain enhances memory formation (Refojo et al., 2011). Conversely, chronic stress and application of CRF at concentrations and durations that reflect chronic stress, result in the retraction of dendritic spines (Chen et al., 2010; Ivy et al., 2010) and loss of LTP (Chen et al., 2013). Activation of the mPFC though CRF signaling mediates both anxiety-like behavior and HPA axis response (Jaferi and Bhatnagar, 2006). Of note, both clinical and rodent research has shown that chronic exposure to stressors creates a shift from declarative to procedural memory. That is, chronic stress suppresses the flexible, cortical-hippocampal-dependent memory and instead favors the habit-like memory mediated by the striatum (Schwabe et al., 2007; Schwabe et al., 2010).

While no other noteworthy changes were observed in the hippocampus, a substantial increase in the excitatory AMPA receptor subunit GluR1 mRNA was observed in the mPFC. Impairment in PFC-function is thought to be especially critical in facilitating drug-addiction. Both opiate and cocaine addiction are associated with impairments in memory, cognitive flexibility and decision-making (Bechara, 2005); neuroadaptations that leave the addicted individual vulnerable to compulsive drug-use and relapse. Indeed, chronic drug use has been shown to lead to increased responsiveness to drug-related cues, and decreased control over drug-taking; behavior that is associated with AMPA mediated increases in glutamatergic transmission from the PFC to the NAc (Kalivas et al., 2005) and dopaminergic cells of the VTA (Saal et al., 2003).

**Ventral Tegmental Area**

The VTA is known for its prominent role in mediating many aspects of drug-taking through strong DA projections to the ventral striatum, NAc and mPFC (Dobi et al., 2010). Dopaminergic firing patterns encode salience of reward, motivation, and the establishment and strengthening of
Consistent with what others have reported, our PCR experiments revealed very little CRH mRNA in the VTA. Instead, the VTA receives CRF projections from the bed nucleus of the stria terminalis, central amygdala and paraventricular nucleus where CRF is co-localized with GABA and Glutamate (Sauvage and Steckler, 2001; Ungless et al, 2003). Accordingly, both CRFR1 and CRFR2 are expressed on DA and non-DA neurons and activation through stress or ICV CRF infusion bring about increases in dopaminergic transmission (Dunn and Berridge, 1987; Koob and Heinrichs, 1999). While, at first glance, our finding of increased CRHbp mRNA in the large suppressor group might seem to indicate a compensatory blunting of otherwise elevated CRF signaling, the role of CRFbp in the CNS (as opposed to its negative regulatory role in the periphery) is more complex (Linton et al, 1990; Ungless et al, 2003). Ungless and colleagues (2003) demonstrated that CRFbp acts in concert with CRF to potentiate NMDAR-mediated EPSCs in the VTA (Ungless et al, 2003). In fact, data suggest that CRF/CRFbp complexes are more potent in potentiating dopaminergic neurons than either protein acting by itself (Ungless et al, 2003).

While many of our findings seem to highlight factors that confer vulnerability to drug addiction, we were intrigued to identify a gene (for β-catenin) whose expression was robustly associated with resilience, or low-drug preference. β-catenin is a protein that serves a dual function; it is a necessary component of cell:cell adhesion, and also activates transcription of the Wnt family of genes, most known for their role in embryonic development (MacDonald et al, 2009). Of further interest, we also found a negative regulator of β-catenin, Cdk5 (Munoz et al, 2007), to be decreased in the VTA of the same suppressors. Together, these would enhance β-catenin signaling and potentially contribute to resilience. Although our qRT-PCR experiments do not elucidate the function of β-catenin, we can speculate that the differential expression of these two genes may act in tandem to promote a greater response to natural rewards. Currently, experiments are being conducted to investigate the role of Wnt signaling in heroin self-administration.
As noted in Figure 6, none of our genes of interest demonstrated significantly different expression levels. On the one hand, this seems surprising given the importance of the NAc in the reward pathway. However, it is gratifying to find that there appears to be anatomical and drug-specific restriction to induced changes in CRF signaling. While increases in NAc-CRF signaling has been observed in psychostimulant administration (Cadet et al, 2014), CRF appears to mediate the increased arousal and motor activity (Holahan et al, 1997) associated with this class of drug. This effect would be expected from an excitatory neurotransmitter in a key motor area, but perhaps not from opioids, which are associated with decreased motor activity (Kosten and George, 2002).

**CpG Islands and Gene Expression**

Early life experiences have been shown to alter CRF expression (Ivy et al, 2010) and the methylation status of the CRH gene (Elliott et al, 2010) and the gene for the CRF receptor (Wang et al, 2013). In this series of experiments, we examined the methylation profiles of genes that: 1) had CpG islands, 2) were significantly different in our qRT-PCR experiments, and 3) demonstrated methylation in preliminary experiments. Within the VTA, we found that at least one CpG in the proximal promoter of the CRHbp gene exhibited less methylation in the large suppressor group, and all but one of the observed CpG methylation sites trended in the same direction. As higher levels of CpG methylation are associated with suppression of gene expression, our results indicate that mRNA expression of CRHbp in the VTA is regulated, in part, by CpG methylation.
Antalarmin

We have previously reported an incubation of heroin-seeking following a period of enforced abstinence (Kuntz et al., 2008b). Our experiments have shown that Antalarmin, a potent and selective CRFR1 antagonist, when delivered peripherally, disrupts heroin-seeking following enforced abstinence. Moreover, this effect is more pronounced in the LS group. CRFR1 antagonists are currently being investigated for the treatment of stress-related disorders such as depression and drug-abuse. Our experiments indicate that the CRF pathway may be involved in various behaviors associated with drug-taking (e.g., escalation, natural reward suppression) as well as relapse-liability. Of note, while it did not reach statistical significance, there appeared to be a rebound effect of Antalarmin delivery on drug-induced reinstatement; animals that received pretreatment with Antalarmin had a stronger reinstatement response than those that received vehicle. This effect was present in both the LS and the SS populations. The half-life of Antalarmin is 28.7 h, so it is likely that Antalarmin is still occupying CRFR1 receptors 7.5 hours after administration. One explanation for this effect is that CRF-CRFR1 signaling mediates drug-seeking (as measured by extinction testing), but is less involved in relapse-liability. Another explanation could be that CRF-CRFR1 signaling blunts perceived saliency of heroin, after the animal is “reminded” of the rewarding effects of the drug (by receiving an injection of heroin).

Limitations

As a caveat, our qRT-PCR experiments, by their nature, have three obvious limitations. First, the tissue from which we extracted RNA (and synthesized cDNA) was from whole brain regions. As each brain region consists of several different cell types, we are not able to ascertain which cells are responsible for our observed differences in mRNA expression (e.g., pyramidal cells vs.
interneurons vs. glia/astrocytes). Secondly, it is widely known that there are regulatory mechanisms that can interfere with the translation of mRNA to protein (Valencia-Sanchez et al., 2006). Our experiments do not assess protein levels; thus, we must be conservative in speculating outcomes of our results. A third and important limitation is that measuring mRNA expression levels of genes only provides information as to the transcriptional milieu at the time of sacrifice. We are unable to ascertain as to whether, for example, the upregulation of CRF signaling components preceded and thus contributed to increased saccharin suppression and heroin intake, or whether the behavior itself (or any behavioral procedures) brought about the changes in mRNA expression. Further, it is possible that any one of the behavioral manipulations employed in our paradigm brought about the gene-expression changes we have reported. In support of this, Williams et al., 2014 recently demonstrated that both self-administration, followed by extinction training, as well as stress-induced reinstatement of cocaine-seeking altered CRF signaling in the VTA (Williams et al., 2014).

Our experiments examining the effect of CRFR1 antagonism on drug-seeking and reinstatement have several limitations. First, our experiment only used 1 CRFR1 antagonist, Antalarmin; other CRFR1 antagonists may bring about different results in similar drug SA paradigms. Second, as we only measured the effect of this antagonist on extinction and reinstatement behavior, the role of CRF signaling in other addiction-like behaviors (e.g., latency to respond, drug-loading, escalation) is yet to be elucidated. Further, given the unexpected response in reinstatement behavior that appears to be related to Antalarmin delivery, future studies should explore the effect of other CRFR1 antagonists on both drug-induced reinstatement, as well as stress and cue-induced reinstatement. Additionally, as our study only utilized a single injection, future studies should also examine the effect of chronic Antalarmin delivery on addiction-like behaviors. Lastly, as our study utilized i.p., peripheral injections of Antalarmin, and not site-specific
injections, it remains unclear as to what particular brain regions are responsible for the effects we observed.

In spite of these limitations, these findings illuminate potential therapeutic targets (CRF signaling components) and/or epigenetic approaches for treating heroin abuse (DNA methylation). Future studies will need to explore the effects of disruption of CRF signaling on natural reward devaluation and increased heroin self-administration.

**Conclusions**

Increases in the expression of CRF signaling components, as well as differences in epigenetic regulatory mechanisms within attention, memory and reward pathways appears to contribute to reward devaluation in heroin SA.
Chapter 3

Effect of Passive Heroin Infusion on Devaluation of Saccharin Cues in Rats: Implications for CRF Systems and Hemispheric Differences


Abstract

As a drug of abuse tightens its hold on addicted individuals, they begin to lose interest in the aspects of life which once brought pleasure (e.g., food, social relationships, job fulfillment/success etc.). Instead, attention and motivation is disproportionately turned toward seeking and acquiring the drug of abuse. In a rodent model of self-administration and reward devaluation, we have previously shown that animals who suppress the intake of a drug-paired saccharin cue show greater addiction-like behaviors, as well as increased gene-expression of the CRF pathway in the mPFC, Hipp, and VTA. In the present study, we explored whether the CRF pathway differences previously observed were a function of self-administration or the devaluation of the saccharin cue. Moreover, as an increasing body of work illustrates both functional and molecular hemispheric differences in reward pathway components, we examined whether the CRF signaling pathway exhibited different expression levels across hemispheres. Over a period of 7 trials, 30 male rats received access to saccharin followed by a passive (IP) injection of heroin (n=20) or saline (n=10). Saccharin intake between large saccharin suppressors (LS) and small saccharin suppressors (SS) were statistically

² This Chapter is comprised of material from a manuscript in final preparation for submission to PLoS ONE. The behavioral passive heroin infusion experiment was designed by PSG, KEV and AJM and was executed by RS and CJ. PSG performed subject sacrifice and AH designed and executed brain dissection (with the aid of AJM). All the molecular experiments were designed and executed by AJM (in consultation with KEV). GB assisted with RNA extraction and RT-PCR. AJM prepared the manuscript and all Figures (with editorial assistance from PSG and KEV). The data in Figure 1 were collected by RS. All remaining data were collected by AJM.
different after the first trial. LS and SS were then assessed for gene expression of the CRF pathway in the mPFC, Hipp, VTA, Amygdala, and NAc. Within the Hipp, LS showed greater expression of corticotrophin releasing factor binding protein (CRFbp). No differences were observed in the mPFC, VTA, NAc or Amygdala. Several hemisphere differences in CRF signaling were also detected. These findings indicate that passive infusion of heroin, at least in this paradigm, does not recapitulate self-administration in terms of CRF pathway expression, and provides further evidence that consideration should be given to hemisphere differences when exploring molecular phenomena.
**Introduction**

Addiction is a disease of brain reward circuitry, and thus, is often accompanied by the emergence of a negative emotional state, excessive attention to drug-associated stimuli and loss of interest in the things of life which once brought fulfillment or pleasure (e.g., food, social relationships, job success etc.). Identifying the neural processes that bring about these changes will aid the discovery of new therapeutics for treating drug addiction.

**Reward Devaluation**

We have previously shown that, in a rodent model of self-administration (SA) and reward-devaluation, animals who suppress intake of saccharin when it predicts the opportunity to self-administer a drug of abuse demonstrate greater addiction-like behavior (e.g., greater drug SA, shorter latency to self-administer drug, greater drug-seeking and greater willingness to work for a drug-infusion) (Imperio *et al*, 2016; McFalls *et al*, 2016). In this model, the saccharin cue may bring about a negative, craving-like state (i.e., rat drug paraphernalia) as suppression of the saccharin reward is also associated with rises in circulating corticosterone (CORT)(Gomez *et al*, 2000), much like what is observed in naloxone-precipitated withdrawal.

**Self-Administration vs. Passive Infusion**

While passive drug infusion is often used as a model of drug abuse, there is substantial evidence to suggest that passive infusion does not recapitulate drug-self-administration. For example, we have previously shown that yoked delivery of cocaine causes greater aversion to a taste-associated cue and avoidance of cocaine-associated regions of the self-administration chamber (Twining *et al*, 2009a). Palamarchouk *et al*. (2009) showed that yoked delivery of cocaine caused greater rises in...
corticosterone levels in the mPFC compared to animals that self-administered the drug (Palamarchouk et al., 2009). As expected, there is evidence to suggest that passive infusion and self-administration bring about differences in neuronal function and activation. Radley et al. (2015) showed that rats that self-administered cocaine had greater reductions in markers of neuronal plasticity in the mPFC (as well as greater deficits in working memory (Radley et al., 2015)). Self-administration of cocaine, but not passive infusion, causes a lasting potentiation of VTA dopamine neurons (Chen et al., 2008). Moreover, human heroin abusers who self-administered the mu opioid receptor agonist fentanyl demonstrated substantially greater delta EEG amplitudes compared to heroin abusers who were given passive delivery of the drug (Greenwald and Roehrs, 2005).

**The CRF Pathway**

We previously investigated the expression of the CRF pathway in rats who suppress a saccharin cue in favor of heroin (large suppressors: LS) and rats who do not suppress saccharin intake and subsequently show little interest in heroin (small suppressors: SS). Heroin self-administration and saccharin suppression were positively associated with greater expression of the CRF pathway in 3 primary regions of the reward pathway: the medial prefrontal cortex (mPFC), hippocampus (Hipp) and ventral tegmental area (VTA). While most known for its role in activating the hypothalamic-pituitary-adrenal axis, CRF orchestrates a global stress-response across the brain and has been shown to be associated with self-administration behavior and increased pain threshold following long-access (6 hour) heroin self-administration sessions (Edwards et al., 2012).
The Case for Studying Hemisphere Differences

There is evidence to suggest that the left and right hemispheres of the reward pathway components have different functions and responses to stress and reward. For example, the mPFC hemispheres differ in their role and response to stress: the right mPFC is implicated in the activation of stress systems, while the left counters this activation through inter-hemispheric inhibition (Sullivan, 2004). Furthermore, the right hemisphere shows higher basal levels of norepinephrine (NE) (Staiti et al., 2011) and also shows greater retraction of spines and dendrites in response to stress (Perez-Cruz et al., 2009). Moreover, several studies have demonstrated hemisphere differences in dopamine (DA) projections to the mPFC (Sullivan and Szechtman, 1994, 1995). A microdialysis study by Staiti (2011) found increased extracellular DA on the right mPFC (Staiti et al., 2011). NE, acetylcholine (ACh) and DA work concurrently in the right basolateral amygdala (BLA) to enhance the retention of emotional memory (in male rats); while blockade of these neurotransmitters in the left BLA has no observed effect on memory (Lalumiere and McGaugh, 2005). There are numerous reports of hemisphere differences in the human hippocampus and other regions (Bottini et al., 1994; Menon et al., 2000) and reports are emerging of hippocampal laterality in animals, as well. For example, optogenetic silencing of the left, but not the right, CA3 region of the hippocampus in mice results in spatial long-term memory deficits (Shipton et al., 2014). Given this, and the known role of CRF signaling to site-specifically modify the function of the reward pathway brain regions, we examined whether CRF signaling components would demonstrate lateral differences in response to a paradigm that presumably involves reward pathway activation. Additionally, given the emerging reports of laterality, we felt it was important to analyze our molecular data with consideration to hemisphere, as it is a standard practice to utilize one hemisphere for a protocol and assume the region is functionally the same on the other side (i.e., perform analysis on one side and confirmation or extension on the other (Kopec et al., 2017; Kremer et al., 2013)).
In the current study, therefore, we: 1) Addressed whether animals would suppress saccharin if it predicted the passive injection of heroin and if the suppression behavior would naturally divide into two phenotypes as previously seen (3); 2) Assessed if passive infusion would recapitulate the individual differences in CRF pathway gene expression that we observed previously in our reward-devaluation and SA model (McFalls et al, 2016), and 3) Determined if CRF pathway gene-expression across the reward pathway (i.e., mPFC, Hipp, VTA, nucleus accumbens (NAc) and amygdala (Amyg)) was different between the left and right hemisphere in response to our behavioral and pharmacological manipulation.
Materials and Methods

Behavioral Analysis

Subjects

The subjects were 30 naïve, male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing between 283 and 323g at the start of experimentation. Rats were singly housed in suspended stainless steel cages in a temperature and humidity controlled animal care facility with a 12 h light-dark cycle (lights on at 0700). Food and water were provided ad libitum, except where noted. All experimental protocols complied with National Institutes of Health Animal Care Guidelines and were approved by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Apparatus and Solutions

All solutions were provided in inverted Nalgene graduated, cylinders with silicone stoppers and stainless steel spouts. Cylinders were attached to the front of each cage with springs. Fluid intake was measured to the nearest 0.5 ml. Sodium saccharin (Fisher Scientific, Malvera, PA) was dissolved in filtered water to a concentration of 0.15% saccharin and presented at room temperature.

Drugs

Heroin was generously provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC) and was dissolved in sterile physiological saline to a
concentration of 8.0 mg/ml. The drug was administered intraperitoneally (IP) at room temperature at a dose of 8.0 mg/kg. The solution was stored at 4°C in a glass vial wrapped in aluminum foil to avoid light penetration and was used within 4 days of preparation.

**Procedures**

Following arrival and one week of environmental acclimation, rats were handled and weighed daily throughout the experiment. After the acclimation period, rats were placed on a water deprivation regimen in which they had 5 min access to filtered water at the front of the cage each morning between 0700 and 0900h and 1 hour access each afternoon between 1500-1700 to maintain hydration. Once morning water intake stabilized, the last day of morning water intake was used to counterbalance rats into two treatment groups: saline (n=10) or heroin (n=20). Twenty-four h later, training commenced and rats were given 5 minutes access to 0.15% saccharin in place of filtered water. After a 5 minute interstimulus interval (ISI), the rats were injected IP with either saline or 15 mg/kg heroin, as per group assignment. There was one such taste-drug pairing every other day for a total of 7 pairings. All rats were given 5 minutes of access to water each morning on the days between conditioning trials and 1 hour every afternoon to maintain hydration.

**Sacrifice and Brain Dissection**

Subjects were sacrificed and brains dissected 48 hours after the last saccharin-heroin pairing (group 1) or on day 7 of the handling procedure (group 2). Sacrifice was performed by rapid decapitation and brains were immediately removed, dissected and then flash frozen for molecular analysis. The following brain regions were harvested as described previously (Heffner, Hartman, & Seiden, 1980): mPFC, containing the prelimbic and infralimbic cortex, (AP: +3.72 to +2.76, from Bregma
based on Paxinos and Watson, 6th Edition, 2007)), NAc, containing the shell and the core (AP: +2.04 to +1.08), Amyg, containing both the central nucleus of the amygdala (CeA) and the basolateral amygdala (BLA) (AP -1.8 to -3.6), Hipp (AP: -4.68 to -6.48), and VTA (AP: -4.68 to -5.4). For each of these regions, the left and right hemisphere were collected in a separate, labeled tube, and the correct hemisphere was confirmed by both the dissector and the person collecting the dissected region.

**Behavioral Statistics**

The saccharin intake data were analyzed using a 3 x 7 mixed factorial Analysis of Variance (ANOVA) varying suppresser group (saline, small suppresser, large suppresser) and trial (1-7) via Statistica 7 (Stat Soft, 2004). Post hoc tests were conducted, where appropriate, using Newman-Keuls tests with α set to 0.05.

**Molecular Analysis**

**Gene Expression**

mPFC, Hipp, VTA, Amg and NAc were analyzed for differential gene expression of the CRF pathway. Genes associated with learning and memory, SA and stress (Deroche-Gamonet et al, 2003; Imperio et al, 2016; Koob et al, 2001; McFalls et al, 2016; Meaney et al, 2002; Nunez et al, 2007) that we previously been found changed in our heroin SA and saccharin suppression model were also assessed for differential expression (Table 1). RNA for gene expression analysis was isolated using the Allprep DNA/RNA kit (Qiagen, Hilden, Germany). Complimentary DNA (cDNA) was synthesized from the isolated RNA using the High Capacity cDNA Reverse
Transcription Kit (Life Technologies, Carlsbad, CA, USA). Messenger RNA (mRNA) expression of CRH and associated genes (Table 1) was assessed though quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The $2^{\Delta \Delta CT}$ method was employed to assess relative gene quantification with beta actin as the endogenous control (VanGuilder et al, 2008). TaqMan gene expression assays were obtained from Life Technologies.
Table 3-1. Genes chosen for qRT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>RT-PCR Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
<td>Rn01462137_m1</td>
</tr>
<tr>
<td>CRHbp</td>
<td>CRH binding protein</td>
<td>Rn00594854_m1</td>
</tr>
<tr>
<td>CRHR1</td>
<td>CRH receptor type 1</td>
<td>Rn00578611_m1</td>
</tr>
<tr>
<td>CRHR2</td>
<td>CRH receptor type 2</td>
<td>Rn00575617_m1</td>
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<tr>
<td>NR3C1</td>
<td>Glucocorticoid receptor (GR)</td>
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<td>Heat-shock protein beta 1</td>
<td>Rn00583001_g1</td>
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<tr>
<td>GRIA1</td>
<td>Ionotopic Glutamate receptor 1</td>
<td>Rn00709588_m1</td>
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<tr>
<td>Rgs4</td>
<td>Regulator of G protein signaling</td>
<td>Rn01490867_g1</td>
</tr>
<tr>
<td>Fgfr2</td>
<td>Beta-Catenin</td>
<td>Rn01269940_m1</td>
</tr>
<tr>
<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
<td>Rn04219635_m1</td>
</tr>
</tbody>
</table>
Subject Selection

Two subjects were not included in the molecular analysis as their saccharin suppression behavior on day 7 did not reflect their behavior in previous trials (i.e., a LS became an SS on day 7, while an SS became a LS).

Molecular Statistics

Gene expression was normalized to the saline group (n=10) so as to visualize the data in a common scale. However, as we were looking at behavioral correlates of gene expression as it relates to drug abuse, this group was not included in further analysis (as saline animals do not have drug-induced behavior). As each brain region was dissected with consideration to hemisphere, each subject in the molecular studies was represented by two values (left and right). In order to test for gene expression differences between LS and SS (without regard to hemisphere), left and right values were averaged for each subject and a standard t-test was utilized to assess gene expression differences between LS (n=10) and SS (n=7) (α=0.05). We employed a two-way ANOVA, varying group x hemisphere, to determine whether there were gene-expression differences between hemispheres in our treatment groups (Saline, LS, SS). Tukey post hoc tests were used to assess differences in gene-expression where main effects were found. Pearson correlational analysis was used to determine the strength of correlation between saccharin suppression behavior and gene expression.
**Results**

**Behavioral Results**

Saccharin intake from terminal trial 7 was used to divide the heroin-treated rats into two groups using a median split. Those rats that suppressed intake of the saccharin taste cue to a larger extent were referred to as the large suppressors (n=11); while those that consumed more of the heroin-paired saccharin cue were referred to as the small suppressors (n=7).

The results of a 3 x 7 mixed factoral ANOVA varying treatment (saline, LS, SS) and trial (1-7) showed that the main effect of treatment was significant, F(2,27) = 28.03, p<0.001, as was the treatment x trials interaction, F(12,162) = 6.25, p<0.001. Newman Keuls post-hoc tests on the treatment x trials interaction revealed the statistically significant differences shown in Figure 3-1. Specifically, while small suppressors significantly reduced intake of the heroin-paired saccharin cue on trials 4 and 6, relative to the saccharin-saline treated controls (p < 0.05), large suppressors significantly reduced intake of the heroin-paired saccharin cue on trials 2 – 7 (p< 0.05). Large suppressor rats also consumed less of the heroin-paired saccharin cue than did the small suppressors on trials 2 – 7 (p < 0.05). As demonstrated previously in our self-administration reward-devaluation model with heroin (Imperio et al, 2015), small suppressors and saline controls were not statistically different in saccharin intake across trials. Table 2 provides a comparison of the self-administration model and the passive infusion model.

**Gene-Expression Results**

**Hippocampus**

Within the hippocampus, LS displayed more mRNA expression of the CRFbp (p<0.05) and CRFR2 genes (p<0.05) (figure 3-2A). We, then, examined this panel of genes between left and right
Two-way ANOVA varying treatment and hemisphere revealed a main effect of treatment \((p<0.05)\) for both CRFbp and CRFR2, but not hemisphere effect or interaction (Figure 3-2B). Posthoc tests using Tukey’s multiple comparison test revealed a significant increase of CRFbp in the LS group vs Sal \((p<0.01)\) and LS vs SS \((p<0.05)\) within the left hemisphere, indicating that the left hippocampal CRFbp may be more responsive to the degree to which a taste cue is suppressed when it is paired with passive infusion of drug. To examine this hypothesis, Pearson’s correlational coefficient analysis was performed on CRFbp gene expression on the left and right vs saccharin suppression on the average of the terminal two trials for both heroin-receiving groups. Left CRFbp \((p=0.04)\), but not right CRFbp \((p=0.38)\), gene expression was significantly correlated with saccharin suppression, with left CRFb expression \((R^2=0.32)\) displaying a stronger association with behavior than right CRFbp gene expression \((R^2=0.08)\). Tukey post hoc testing revealed that CRFR2 was significantly decreased in the SS group relative to Sal in the Hippocampus in the right hemisphere \((p<0.01)\) (Figure 2B).

**mPFC**

No differences in CRF pathway gene-expression were observed in the mPFC between LS and SS when hemispheres were combined (Figure 3A). Additionally, there were no differences found between groups when comparing left and right hemisphere (Figure 3B). We also examined the expression of the gene Gria1 (as we had previously observed differential expression between LS and SS (McFalls et al, 2016)) and found no differences between groups as well as between hemispheres (data not shown).
**VTA**

Within the VTA, we found no overall differences in CRF pathway gene expression between LS and SS (Figure 3-4A). However, when we examined CRF pathway gene-expression between the left and right hemisphere, two-way ANOVA revealed a significant main effect of group, but no significant main effect of hemisphere or group x interaction effect. Tukey post hoc testing showed that SS had significantly less mRNA expression of CRF in the left hemisphere compared to saline controls (p<0.01) (Figure 3-4B). Additional gene expression analysis was conducted for RGS4, Cdk5 and Fgfr2 (as we had previously observed differential expression of these genes between LS and SS (McFalls et al, 2016)). No differences were found in regard to group or hemisphere (data not shown).

**NAc**

No differences in CRF pathway expression were found between LS and SS in the NAc (Figure 3-5A). Additionally, the comparison CRF pathway gene-expression across hemispheres using two-way ANOVA showed no significant effects of group, hemisphere or group x hemisphere interaction (Figure 3-5B).

**Amygdala**

We observed no differences between LS and SS CRF pathway gene-expression within the amygdala (Figure 3-6A). Additionally, two-way ANOVA revealed no significant effects of group, hemisphere or a group x hemisphere interaction (Figure 3-6B).
Figure 3-1. – A subset of rats suppress saccharin intake when it is paired with a passive delivery of heroin.

Rats were given the opportunity to ingest saccharin 5 minutes prior to receiving an injection of heroin (15 mg/kg) or saline. Saline (n=10), small suppressor (n=8), large suppressor (n=12). ### denotes p<0.001 between saline and large suppressor, *** denotes p<0.001 between small suppressor and large suppressor
Figure 3-2. – CRF pathway component expression in the Hippocampus.

A. Independent t-tests revealed more gene-expression for CFRbp and CRFR2 in LS compared to SS. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.  B. Two-way ANOVA revealed a group effect for CFRbp mRNA expression. LS had approximately a 50% increase of CFRbp in the left hemisphere compared to saline, and SS had approximately a 45% increase. Two-way ANOVA revealed a group effect for CRFR2 expression. SS had approximately a 60% decrease of CRFR2 gene expression compared to saline in the right hemisphere. * denotes p<0.05, ** denotes p<0.01. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.
Figure 3-3. – CRF pathway component expression in the mPFC.

A. Independent t-tests revealed no gene-expression differences between LS and SS. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.

B. Two-way ANOVA revealed no group or hemisphere differences of CRF component expression in the mPFC. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.
Figure 3-4. – CRF pathway component expression in the VTA.

A. Independent t-tests revealed no gene-expression differences between LS and SS. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.

B. Two-way ANOVA revealed a group effect between saline and SS of CRF mRNA expression. ** denotes p<0.01. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.
Figure 3-5. – CRF pathway component expression in the NAc.

A. Independent t-tests revealed no gene-expression differences between LS and SS. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.

B. Two-way ANOVA revealed no effect of hemisphere or group within CRF signaling genes. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.
Figure 3-6. – CRF component expression in the Amygdala.

A. Independent t-tests revealed no gene-expression differences between LS and SS. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.

B. Two-way ANOVA revealed no hemisphere or group effect. Boxes represent median (line) and 1st and 3rd quartile, whiskers represent minimum and maximum values.
Table 3-2. Comparison of self-administration experiment (Chapter 2) and passive infusion experiment (Chapter 3).

<table>
<thead>
<tr>
<th></th>
<th>Self-administration</th>
<th>Passive infusion</th>
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</thead>
<tbody>
<tr>
<td><strong>Route of administration</strong></td>
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<td>intraperitoneal</td>
</tr>
<tr>
<td><strong>Dose</strong></td>
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<td>8 mg/kg</td>
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<tr>
<td><strong>Infusions terminal trial</strong></td>
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<tr>
<td><strong>Trials</strong></td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td><strong>Progressive ratio testing</strong></td>
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<td><strong>Extinction testing</strong></td>
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</tr>
<tr>
<td><strong>Drug-primed reinstatement</strong></td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><strong>Avoidance of drug-associated taste cue</strong></td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Large and small saccharin suppressors</strong></td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Time from last infusion to sacrifice</strong></td>
<td>24 hours</td>
<td>48 hours</td>
</tr>
</tbody>
</table>
Here, we show that approximately half of an outbred group of male Sprague-Dawley rats suppress saccharin intake when it precedes a passive infusion of heroin. However, while we demonstrate marked individual differences, not unlike those obtained following saccharin paired with heroin self-administration, we also show that mRNA expression of the CRF pathway components with passive infusion is markedly different from that found in our self-administration model (Imperio et al., 2015; McFalls et al., 2016). Table 2 provides a comparison of the self-administration model and the passive infusion model used herein. Finally, we also provide evidence that left and right hemisphere gene-expression of CRF pathway components is differentially responsive to behavior and changes in the pharmacological environment.

**Passive Infusion vs Self-Administration**

Although passive infusion of drugs of abuse is commonly used to study the pharmacological effects of drugs of abuse (Kim et al., 2000; Lee et al., 2000; Williams et al., 2012), there is a growing body of evidence that suggests that passive infusion does not recapitulate SA (Caffino et al., 2014; Chen et al., 2008). Drug addiction in humans involves the complex orchestration of behavior (i.e., habits, responses to external and internal cues) learning and memory (i.e., learned associations), as well as pharmacological manipulation of brain chemistry. Accordingly, several laboratories have shown that even the molecular effects of a given drug are different in the absence of behavior. Chen et al. (2008) reported that SA of cocaine caused a persistent potentiation of VTA neurons, but this effect was lost when the cocaine was experimenter-delivered (Chen et al., 2008). Caffino et al. (2014) found that alpha calcium/calmodulin-dependent protein kinase type II (previously shown to mediate the motivation to seek and self-administer cocaine (Li et al., 2008)) was elevated in the
mPFC and NAc of rats that self-administered cocaine, but not those who received passive cocaine infusions (Caffino et al, 2014). It is not surprising, therefore, that we also found different molecular responses between passive infusion and SA. Specifically, we previously reported that self-administration was associated with increased gene expression of CRFR1 in the mPFC and Hipp, and increased CRFbp (as well as differential expression of beta catenin, Cdk5 and Rgs4) in the VTA (McFalls et al, 2016). In the present study, the expression of only two genes CRFbp and CRFR2 was altered in a single brain region in the hippocampus. Interestingly, both of these genes were expressed in the same direction as our previous report, which may indicate that these genes may play a role in drug-associated saccharin suppression and highlight the role of hippocampus in that process.

The Extrahypothalamic CRF Pathway

Previously, we have shown that greater avoidance of a saccharin cue paired with the opportunity to self-administer heroin is associated with increased gene expression for the CRF pathway components across the reward pathway (McFalls et al, 2016). In this set of experiments, we show that passive infusion of heroin (and avoidance of the associated taste cue) is associated with a different gene expression pattern of the CRF pathway components throughout the reward pathway than we have previously seen in our heroin SA model. The extrahypothalamic CRF pathway orchestrates a global stress response across the brain, acting upon different sites to bring about appropriate responses to a perceived threat. Further, both of its G-coupled receptors, CRFR1 and CRFR2 (Perrin et al, 1999), have several splice variants which are distributed differentially throughout the brain, resulting in a diverse and tissue-specific response (Refojo et al, 2011). For example, in the hippocampus, CRF is released from basket cell interneurons onto glutamatergic pyramidal neurons (Refojo et al, 2011) and dose-dependently enhances memory formation (Refojo
et al., 2011) and LTP (Chen et al., 2001) (of note, similar to the effects of glucocorticoids, sufficiently high levels of CRF cause a reduction of memory via retraction of dendritic spines (Chen et al., 2010; Ivy et al., 2010) and loss of LTP (Chen et al., 2013)). In the VTA, CRF (through stress or ICV CRF infusion) acts upon DA neurons to enhance dopaminergic transmission (Dunn et al., 1987; Koob et al., 1999).

Previously, we reported CRFR1 mRNA to be elevated in the mPFC of saccharin-suppressing, heroin-preferring rats (McFalls et al., 2016). In the current study, we observed no changes in CRF pathway mRNA expression in the mPFC. There are several things that could account for this difference. CRFR1 mRNA expression has been shown to increase in response to stress, and CRF-CRFR1 transmission in the mPFC alters executive function (Uribe-Marino et al., 2016). In this study, as there was no “decision” made regarding how much heroin to self-administer, it is possible that CRF signaling in the mPFC mediates, in part, SA behavior (see McFalls et al., 2016). Further, both the route of administration and dose used in this study were different from our SA paradigms (see “limitations”). As the rats in this study received less heroin over fewer trials, although speculative, it is also possible that they did not experience a stressful withdrawal-like state (that would accompany higher doses) and thus, did not experience a robust activation of the CRF pathway.

Using our reward-devaluation SA model, we have also reported increased expression of CRFR1 in the hippocampus of saccharin-suppressing, heroin-preferring rats (McFalls et al., 2016). Here, we observed increased expression of CRFbp and CRFR2 in the hippocampus of the LS group. This is an interesting finding as both CRFbp and CRFR2 act somewhat in opposition to CRF-CRFR1 signaling in the hippocampus. As mentioned previously, CRF-CRFR1 activation in the hippocampus is a component of stress-related memory formation and anxiety behavior (Janssen and Kozicz, 2013; Refojo et al., 2011). In the hippocampus, CRFbp is expressed in neurons in the entorhinal cortex and may serve to limit the effect of CRF (Park et al., 2003). The principal ligand
for CRFR2 is not CRF, but rather, the urocortin family (UCN-I, UCN-II and UCN-III). Although there is much to be discovered regarding CRFR2 signaling in the brain, it is thought to mediate stress-recovery (Henckens et al., 2016; Janssen et al., 2013). Although we cannot perform a direct comparison, CT values can be used to approximate relative levels of a gene’s expression. Using a comparison of CT values across groups, we estimate that CRFR1 (CT average = 30.0) is expressed 3.5 times more than CRFR2 (CT average = 31.9) in the hippocampus. This agrees with our previous data (approximately 2.5 times more CRFR1 than CRFR2), as well as others (Henckens et al., 2016; Van Pett et al., 2000), and likely indicates that CRF signaling in the hippocampus is primarily mediated through CRFR1.

Another interesting and unexpected finding from this study was the lack of differences in CRF component pathway gene expression in the amygdala. The CRF system in the amygdala has gained increasing attention in the drug-abuse field. CRF signaling in the amygdala has been found to play a central role in addiction to alcohol (Edwards et al., 2012; Lowery-Gionta et al., 2012), opioids (Edwards et al., 2012; McFalls et al., 2016; Park et al., 2013) psychostimulants (Erb et al., 2001) and even palatable food (Cottone et al., 2009). In the current study, we found no differences in CRF pathway gene expression between LS and SS. Again, this difference could be due to the short duration of our study and/or the lower dose of heroin failing to produce dependence and acute withdrawal states that are postulated to bring about recruitment of the CRF system (Koob, 2010). In support of this, Heilig et al. (2010) posit that CRF signaling does not play a vital role in the initial, pre-dependent state in ethanol SA (Heilig et al., 2010). Rather, Koob maintains that CRF signaling in the extended amygdala mediates the transition to dependence and maintenance of the addicted state (Koob, 2009a). Alternatively, as the amygdala is known to play a role in memory formation and motivation, it is possible that the opportunity for drug-seeking, taking and cues surrounding self-administration all conspire to increase CRF signaling in the amygdala (all things that are lacking from a passive infusion paradigm). Another possible reason for the lack of
differences observed in our study could stem from our inclusion of both CeA and BLA in the amygdala analysis. The BLA has been implicated as a key site in the association of conditioned and unconditioned stimuli and the CeA is generally thought of as an output region of BLA activity (Jimenez and Maren, 2009; Zimmerman et al., 2007). Accordingly, these two areas may respond differently to the behavioral and pharmacological changes in our model. Thus, the inclusion of both regions in one analysis may have precluded the observation of gene expression changes. Future studies should examine reward devaluation on both the CeA and the BLA, separately.

**Hemisphere-Specific Expression of CRF-Signaling Molecules**

Reports are emerging of laterality in the function and chemistry of various brain regions. This is the first time, to our knowledge, that CRF pathway mRNA expression was examined in regards to left and right hemisphere in the reward pathway. We found that, within the hippocampus, CRFbp expression was higher in LS on the left, while CRFR2 expression was higher in LS on the right. CRFbp gene expression in the left hemisphere was reminiscent of a dose effect, in that greater saccharin suppression was associated with increased CRFbp gene expression. Although the strength of correlation was not overwhelming, several factors including the short duration of the study (and, thus, a brief period of saccharin suppression) may account for this. Future studies should examine left hippocampal CRFbp gene expression in longer paradigms that may induce greater saccharin suppression, as well as examining hemispheric analysis of hippocampal CRFbp in reward devaluation, self-administration paradigms. Hemisphere differences were also found in the VTA, where CRF expression was lower on the left side in the SS group. Hemisphere differences have been found in regards to DA projections from the VTA to the mPFC (Sullivan et al., 1994, 1995) and DA release from the VTA is modified by CRF signaling. In fact, CRFR1 knockout on DA neurons produces an increase in anxiety-like behavior and decreased stress-
mediated DA release in the mPFC (Janssen et al., 2013). Grieder et al. (2014) identified a small population of DA neurons in the bilateral posterior VTA that synthesize CRF and locally release CRF onto posterior VTA GABAergic neurons (that, in turn, inhibit VTA DA neurons). Increased CRF mRNA synthesis in these neurons was associated with nicotine dependence and withdrawal (Grieder et al., 2014). Interestingly, in our study, rats who received passive heroin had lower levels of CRF mRNA than their saline receiving counterparts. Again, it is possible that the short duration of our study was not sufficient bring about dependence and withdrawal. Still, the pattern of expression was opposite from what we expected. Taken together, our studies indicate that the hemispheres may respond differently to behavioral paradigms and/or drug delivery that may have a stress component.

Limitations

One major limitation of this study was that the route of administration of heroin was different from our previously published study showing differential CRF pathway expression in a model of reward devaluation and SA. IP injection and intravenous (IV) injection have markedly different pharmacokinetics. For example, the ED$_{50}$ value for the analgesic effect of morphine (in mice) is 8.9 mg/kg IP, while the IV ED$_{50}$ is 6.7 mg/kg (Tokuyama et al., 1993). The bioavailability of dihydroetorphine (DHE), a potent $\mu$ opioid receptor agonist, injected IP was found to be 16.7% of DHE injected IV (100%). In our current study, our rats received one IP injection of heroin (8mg/kg) per trial over a period of 7 trials. In contrast, in our SA study, each infusion IV was 0.06mg/0.2ml heroin over a 6 hour period, in which the animals took approximately 20 infusions in the last trials (Imperio et al., 2015). Future studies should examine CRF signaling gene expression in a passive, yoked model that would provide identical dose and route of administration and also hemisphere differences of CRF signaling in SA models.
Conclusions

Here, we show that approximately half of animals will suppress saccharin intake when it predicts passive heroin delivery. We also report that animals that respond differently to a heroin-associated taste cue in a passive infusion model demonstrate a different profile of CRF signaling component gene-expression than in our previously reported SA model (McFalls et al, 2016). In particular, no differences between LS and SS animals was observed in the mPFC, NAc, Amygdala and VTA, areas where we would expect to see differences. This may be due to the absence of “decision-making” (mPFC) tasks in regards to heroin SA and the motivation (NAc and VTA) to seek heroin that is inherently present in a SA model. Lastly, this is the first report of hemisphere specific differences in CRF signaling gene-expression throughout reward pathway components. Taken together, our data further demonstrate that passive infusion models bring about different molecular effects than SA, and that researchers should give consideration to hemisphere when examining molecular phenomena.
Chapter 4

An RNA-seq Study of the mPFC of rats with Different Addiction Phenotypes

McFalls AJ, Imperio CG, Wronowski B, Grigson PS, Freeman WM, Vrana KE

Abstract

Addiction is a disease of brain-reward circuitry whereby attention, motivation, memory and emotional systems become enslaved to the goal of seeking and acquiring drug, instead of responding to the natural rewards for which these systems evolved. At the intersection of reward/limbic structures, the mPFC receives and consolidates signals regarding environment and orchestrates the most appropriate response (i.e., decision-making and attention). As such, mPFC function plays a critical role in the vulnerability or resilience to drug addiction. In our model of drug-induced reward devaluation, an outbred group of Sprague-Dawley rats parsed into two distinct drug-taking phenotypes: those, referred to as small suppressors (SS) that readily ingest a heroin-associated sweet cue, and those, referred to large suppressors (LS), that avoid the heroin-associated cue, but then respond greatly for the drug of abuse. In the present study, we analyzed the mPFC transcriptome of rats of these divergent phenotypes in order to discover gene expression differences that underlie these phenotypes. Genes found to be differentially expressed were those associated with schizophrenia and dopamine signaling, signal transduction, development and synaptic plasticity. These genes may underlie the phenomena whereby some individuals succumb to addiction, while others do not, and may provide new pharmacological targets for the treatment of drug addiction.

3 This Chapter is comprised of material from a manuscript in preparation for submission. The initial behavioral self-administration of heroin experiment was designed and executed by CGI (in consultation with PSG). All the molecular experiments were designed and executed by AJM (in consultation with WMF and KEV). BW performed library preparation and next generation sequencing (NGS). WMF assisted with NGS data analysis instruction. All Figures were generated by AJM (with editorial assistance from KEV).
Introduction

Heroin Addiction and Reward Devaluation

Addiction is a complex individual and societal problem characterized by loss of control over drug-seeking and taking and chronic relapse. Perhaps what is most puzzling to those that do not have an addiction is the degree to which addicted individuals will cause damage to their lives (and the lives of those around them) in order to continue drug use. Salient examples of this include the loss of job and/or home, neglect of offspring (Nair et al, 1997), and stealing from relatives or place of employment in order to buy more drug (Statistics, 2004). On the outside, it is easy to see this kind of behavior as a moral failing when, in fact, it is a reflection of maladapted brain-reward circuitry – a phenomenon that can be modeled in the laboratory. The most rewarding things of life (e.g., highly caloric food, social relationships, achievement, sex), are the very things that promote the survival of individuals, species and genes; therefore our brains are uniquely primed to respond to these rewards. The experience of natural rewards causes the release of dopamine from neurons of the VTA that project to the NAc (motivation), hippocampus (memory formation), mPFC (attention and decision-making) and amygdala (valence), and brings about current and future procurement of “life-giving” rewards. Drugs of abuse are addictive, in part, because they also utilize these pathways, but do so to a supra-physiological extent, tricking the brain into thinking that the drug, not natural rewards, are the most “life-giving” rewards. As a result, natural rewards lose their ability to elicit the neural response that they once did, and, instead, the addict is now primed to respond preferentially to drug cues.
The mPFC

The medial prefrontal cortex is a brain region that influences, and is affected by, the process of addiction. The mPFC is a brain region with substantial afferent and efferent connections with limbic structures. Accordingly, it is known for its prominent role in attention and decision-making as well as the inhibition of impulses and emotion. However, as our understanding of connections and functions increases, the mPFC might be more accurately described as a region that makes associations between context, memory and emotional valence and selects the most appropriate response based upon that criteria (Euston et al, 2012).

Given its role in behavior, it is perhaps not surprising that the mPFC plays a substantial role in the progression and maintenance of drug addiction. Chronic drug use alters the function of the mPFC, bringing about both decreased ability to control impulses and craving (hyporesponsiveness) and increased attention to drug-associated cues (hyper-responsiveness) (Robinson et al, 2008). Together, these adaptations leave the addicted individual at a substantial disadvantage for overcoming addiction.

Reward-Devaluation Model

We have developed a rodent model of the devaluation of natural rewards in which a rat is able to administer both saccharin, a sweet palatable substance, and then a drug of abuse. The animal is therefore able to choose its level of intake of the “natural” reward cue and of the drug of abuse (Imperio et al, 2015). In brief, rats are given 5 min access to 0.15% saccharin and immediately thereafter 6 h in which to intravenously (IV) self-administer heroin (0.06 mg/inf). We have previously shown that when an outbred group of Sprague-Dawley rats is placed in this paradigm, two distinct phenotypes emerge: Small Suppressors (SS), who, like the saline controls, increase
their intake of saccharin over trials and Large Suppressors (LS) who avoid the saccharin cue over trials. Interestingly, the suppression level of saccharin predicts drug taking behavior; LS show shorter latency to self-administer, greater loading behavior and greater drug intake (i.e., large drug takers), while SS demonstrate “resilience” to these addiction-like behaviors (i.e., small drug takers) (Freet et al., 2015; Grigson, 2008; Imperio et al., 2015). Interestingly, in this model, saccharin, although palatable to rats not ingesting a drug of abuse, begins to resemble a sort of rat-drug-paraphernalia whereby this once rewarding substance now brings about rises in circulating corticosterone (CORT) (Gomez et al., 2000) and negative orofacial responses (Wheeler et al., 2008), an effect that mirrors those produced by naloxone-induced withdrawal (Nunez et al., 2007).

**RNA-Seq Analysis and BSAS**

Previously, we have reported a number of gene expression differences between animals of different heroin-taking phenotypes. Our recent RNA-Seq analysis of the nucleus accumbens revealed differential expression of genes related to learning and memory, neuronal outgrowth, neuronal stimulation, movement disorders and immune function and signaling in LS vs. SS rats (Imperio et al., 2016).

In light of the mPFC’s central role in reward procurement, this study examined the transcriptome of animals with distinct heroin-taking phenotypes: large and small saccharin suppressors (large and small heroin takers, respectively). Additionally, it is now recognized that the regulation of genes at the epigenetic level changes in the adult organism in response to environmental cues. Therefore, we investigated epigenetic regulation of differentially expressed genes through BiSulfite Amplicon Sequencing (BSAS): a next generation sequencing technique that allows for absolute quantification of methylated/unmethylated cytosines at multiple gene locations.
Materials and Methods

Behavioral Paradigm

Subjects and Surgeries

Twenty male Sprague-Dawley rats, obtained from Charles River at approximately 90 days of age were the subjects of this study. Rats were individually housed in stainless steel suspended cages in a humidity controlled environment and maintained on a 12/12 h light/dark cycle. All rats were weighed daily and food and water was available ad libitum, except where otherwise noted. For catheter and cannula implantation, each rat was anesthetized with a mixture of ketamine (70 mg/kg, ip) and xylazine (10 mg/kg, ip) and a catheter was implanted into the right jugular vein as previously described (Colechio et al, 2014; Puhl et al, 2013; Twining et al, 2009b).

Apparatus

Saccharin and heroin self-administration sessions took place in 12 self-administration chambers as described previously (Puhl et al, 2013). Each chamber was equipped with a house light (25W), a tone generator (Sonalert Time Generator, 2900 Hz, Mallory, Indianapolis, IN), a speaker for white noise (75 dB) and 3 retractable spouts. Spout contacts were recorded by a lickometer (a contact relay circuit) and a stimulus light was located above each spout. The leftmost spout delivered saccharin, the middle spout was “inactive” (no amount of licking resulted in an outcome), and the rightmost, “active” spout was on a FR10 schedule of heroin delivery (10 licks resulted in an infusion of heroin). All activity in the self-administration chamber was recorded and collected by a computer using Medstate notation language (MED Associates, Inc., St. Albans, VT).
Drug Acquisition

During habituation, rats were placed in the self-administration chambers for 5 minutes a day, over a period of 3 days. In order to train the rats to lick the spouts, they were placed on a water restriction regimen (25 mL of water in the home cage overnight) and were then given access to water through 1 of the 3 spouts during the 5 minute habituation sessions. After habituation, the rats began the drug acquisition period. During this phase of the study, rats were given access to a 0.15% saccharin solution in the first five minutes, followed by a 6 hour self-administration session in which the rats could work for infusions of heroin (0.06mg/0.2ml) (n=13) or saline (n=7). The acquisition period occurred 5 days a week for a total of 16 trials.

Progressive Ratio Challenge

On the 17th trial, willingness to work for heroin was examined by a progressive ratio (PR) test. Rats were given a 5 min access period to saccharin as in previous trials and were then placed on a PR schedule of reinforcement for heroin. In this test, successive IV infusions of drug depended upon making an increasing number of contacts with the empty spout operant according to the following schedule: 10, 10, 12, 16, 22, 30, 40, 52, 66, 82, 100, 120, 142, 166. Breakpoint was defined as the last ratio completed. The trial ended when 30 min elapsed without the animal having earned an infusion (Imperio et al, 2015; Puhl et al, 2009).

Extinction/Reinstatement

On the 18th trial, the rats were presented with an extinction/reinstatement test (E/R) in order to assess drug-seeking behavior. During E/R, the rats were first given 5 minutes access to saccharin, as before. After this, the animals received the cue that heroin was available (i.e., the saccharin
spout retracted, and the inactive and active spouts advanced). During extinction, however, no amount of licking on the active spout resulted in an infusion. This assessment of seeking lasted 6 hours. In the subsequent reinstatement phase (hour 7), the animals were then given one infusion of heroin and heroin seeking was again assessed for one hour.

**Behavioral Stratification**

Rats were behaviorally stratified based on saccharin suppression (saccharin licks) on the terminal trial (trial 16) as previously described (Grigson and Twining, 2002). Rats with >200 licks/5 min saccharin session were deemed Small Suppressors (SS). Rats with <200 licks/5 min were deemed Large Suppressors (LS).

**Gene Expression Analysis**

**Sacrifice and Tissue Dissection**

Rats were sacrificed by rapid decapitation 24 hours after the E/R session to ensure that no drug was in their systems at the time of sacrifice so as to assess gene expression changes not engendered by acute drug administration. The mPFC was dissected as previously described (Kasprowicz et al, 2008), flash frozen and stored at -80°C for further molecular analysis. Of note, one hemisphere was utilized for RNA-Seq analysis, while the opposite hemisphere was used for orthogonal QRT-PCR confirmation. In both cases, the tissues used in these analyses were presumably a mix of left and right mPFC, with the assumption that gene expression is hemispherically comparable.
Subject Selection Criteria for RNA-Seq Analysis

Subjects for analysis in the RNA-Seq experiment were chosen based on greatest saccharin intake (SS, n=5) or greatest saccharin suppression (LS, n=5) in trial 16, and RNA quality (RNA integrity number (RIN) > 8) (The RIN is based on the ratio of 28S to 18S rRNA in an electropherogram trace; generally, an RIN of >7 is considered good quality RNA). One SS sample was removed from the molecular studies based on poor read quality in the RNA-Seq experiment. Five saline animals were randomly chosen as controls.

RNA Isolation and cDNA Synthesis

DNA and RNA were isolated from mPFC tissue using the Allprep DNA/RNA isolation kit (Qiagen) and complementary DNA (cDNA) was generated from 1 µg RNA with the ABI High Capacity cDNA Reverse Transcription Kit (Life Technologies). RNA quality and quantity were assessed using the RNA 6000 Nano Assay with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

Library Construction and Next Generation Sequencing

Libraries were constructed using the Illumina Truseq Stranded HT library kit, an approach that retains the directionality of transcripts. Briefly, oligo-dT beads captured and purified polyadenylated tails of mRNA. mRNA was then chemically fragmented upon elution and cDNA was synthesized. In order to maintain directionality of the transcripts, dUTP (instead of dTTP) is incorporated into the second strand during cDNA synthesis; the polymerase is unable to read past this nucleotide. After synthesis, cDNA products underwent end repair, the addition of a single “A” base, and ligation of the adapters. The cDNA products were then purified for sequencing and enriched using PCR to make a library for sequencing. Libraries were quantified by qRT-PCR.
(Kapa Biosystems) and sized by TapeStation (Agilent). The cDNA libraries were then sequenced on an Illumina Hiseq2500 (2x100bp).

**RNA-Seq Data Analysis**

After sequencing, reads were aligned, filtered, trimmed and analyzed for differential expression using the strand NGS software package (Agilent). Reads were aligned to the Rnor 6 build of the rat genome. Alignment and filtering included the following read quality metrics: 2 bp were trimmed from the 3’ and 5’ end of each read, low quality reads were trimmed off the 3’ end (Q<20), duplicate, ambiguous or reads in the wrong direction were filtered out, and valid reads required an 80% minimum match length with the reference sequence. Reads were aligned in a directional manner with read 1 in the reverse direction, and read 2 in the forward. Normalization was performed using Reads per Kilobase per Million Mapped (RPKM), Trimmed Mean of M-values (TMM) and Differential Expression-seq (DEseq). Three lists of differentially expressed genes were generated for each normalization method: Saline vs. SS, Saline vs. LS and SS vs. LS. The lists from each normalization method were then overlaid, an only genes found to be changed by all three normalization method were chosen for further analysis (e.g., Saline vs. SS: RPKM/TMM/DEseq) (with the assumption being that a gene found to be changed by all 3 normalization methods is likely not an artifact). One-way ANOVA (p<0.05) and Student-Newman Keuls post hoc test were utilized to discover differentially expressed genes between the three experimental groups. A 1.25 fold cutoff was further employed to isolate genes of physiological relevance (not just statistical significance).
**Gene Selection Criteria**

Genes selected for further qRT-PCR confirmation were those that were found to be differentially expressed in all three normalization methods in the RNA-Seq analysis (see Figure 1). Additional selection criteria included the selection of genes whose function had known ties to brain function and/or behavior (see Table 1). For example, as heroin is a known modifier of the immune system, many immune genes were found to be differentially expressed between the three groups, as previously reported (Imperio *et al.*, 2015). These immune genes were not explored further in the present study but are provided in publically available data sets in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo). However, analysis of immune gene expression and drug-taking behavior revealed this to be a dose effect of heroin on the immune system (data not shown). Additionally, potential disease relationships, functions, signaling pathways and upstream regulators were explored using the Ingenuity Pathway Analysis (Qiagen, Redwood City, CA) database.

**Confirmatory qRT-PCR**

Genes found to be differentially expressed were assessed by the orthogonal method, qRT-PCR using TaqMan assay on demand prime/probe sets (see Table 1 for gene and primer list) and a QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems) according to our standard methods (Bixler *et al.*, 2011; Masser *et al.*, 2014). Relative gene expression was calculated with ExpressionSuite v 1.0.3 software using the 2−ΔΔCt analysis method with β-actin as an endogenous control. qRT-PCR confirmation analysis was performed in the same subjects used in the RNA-Seq analysis (n=5/group). Independent t-tests were used to compare results from qRT-PCR analysis based on prior differences found using RNA-Seq. Of note, due to tissue quantity restrictions, the
opposite hemisphere was used for orthogonal qRT-PCR confirmation (with the assumption that gene expression across hemispheres would be comparable).

Methylation Analysis

*Genomic Repeat Element Analysis*

Methylation levels of repetitive elements were examined as a surrogate for genome-wide methylation changes. Genomic DNA (n=2-5/group) was bisulfite-converted using the EZ DNA methylation-lighting kit (Zymo Research, Irvine, CA) and then subjected to PCR amplification using bisulfite-specific converted primers for LINE-1 and ID elements (EpigenDx, Hopkinton, MA). Following PCR amplification, amplicons were analyzed for CpG methylation via pyrosequencing (EpigenDx, Hopkinton, MA). Methylation levels across groups for LINE-1 and ID elements were analyzed using one-way ANOVA (Figure 4-5).

*Gene Selection*

Genes for further methylation analysis were chosen on the basis of 1) demonstrating differential expression in the RNA-Seq analysis using all 3 normalization methods, 2) differential gene expression was confirmed using qRT-PCR, and 3) the gene promoter, exon 1 and/or intron 1 must contain CpG islands (i.e., CpG observed/CpG expected ≥ .6 in a 100-2000 base-pair region); (thus indicating that the expression of the gene may be regulated by cytosine methylation). Five out of the ten chosen genes met these criteria: Adora2a, Arhgef28, Bche, Fxyd6 and Pcdhga5.
**Primer Design**

Primers were designed to target CpGs in regulatory regions of genes (i.e., promoter, exon 1 and intron 1) (Figure 6, Table 2). Gene sequences were obtained from the ENSEMBL genome browser and CpG islands were calculated and primer suggestions were generated using the Methyl Primer Express Software v1.0. Primers were then modified so that: 1) the 3’ end terminated in a guanine (forward primer) or a cytosine (reverse primer) (so as to increase the binding strength of the polymerase to the DNA); 2) the Tm of the forward and reverse primers were within 1.5 C; and 3) the primer did not contain large stretches of thymine (as bisulfite-treated DNA is extremely homogeneous after the C to T conversion). Primers were designed to not contain or be directly adjacent to a CpG sequence, as this is prone to generating a false signal (Masser *et al*, 2013). Primer sequences are provided in Table 2. Primers were amplified using bisulfite-treated DNA, resolved on a 2% agarose gel with ethidium bromide and visualized using a UVP Bio Dock-it system. Amplicons were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequences were confirmed via traditional sanger-sequencing.

**NGS Library Preparation**

Amplicons were generated, purified using a Zymo Clean and Concentrate-5 kit (Zymo Research, Irvine, CA), and resolved on a 2% agarose gel to confirm correct amplicon size. Amplicons were then isolated from the gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and quantified using PicoGreen (Promega, Madison, WI). Amplicons were diluted to 0.2 ng/μl and pooled (i.e., 16 amplicons in/for 1 sample) and a total of 3.2 ng was used for library generation. Dual indexed libraries were generated using Nextera XT library prep kit according to manufacturer’s instructions.
Results

Drug-Taking Behavior

A detailed report of drug-taking behavior can be found in Imperio & Grigson (2015) (Imperio et al., 2015). In brief, rats who continually increased intake of the saccharin cue (small suppressors) over the 16 trials showed modest heroin intake, while those who avoided intake of the saccharin cue (large suppressors) escalated heroin intake over the 16 trials. Figure 1 depicts saccharin-taking and heroin SA of the rats chosen for this study (n=4-5/group). Large suppressors took significantly less saccharin than small suppressors from trials 9-16 (Figure 4-1A). Heroin self-administration was greater in the large suppressor group relative to the small suppressor group in trials 9-10 and 12-16 (Figure 4-1B). Heroin self-administration was significantly increased in the large suppressor group when comparing trial 1 to trial 16 (p>0.05). Small suppressors, conversely, did not significantly increase heroin intake over time. Large suppressors also worked significantly harder to obtain drug in the PR trial, and displayed more drug-seeking behavior in the E/R trial (as detailed in (Imperio et al., 2015)).

RNA-Seq Analysis

Differentially expressed transcripts between groups were identified though a one-way ANOVA design with Student Neuman Keuls pair-wise post hoc test (α<0.05). These transcripts were further filtered to only those that had a >=1.25| fold difference between the groups that had a significant post-hoc test result. This resulted in a total of 587 differentially expressed transcripts between the three experimental groups of the 31,399 transcripts with rat RefSeq annotations. Differentially expressed transcripts were further analyzed using 3 different normalization methods: RPKM,
Deseq, and TMM and 3 different comparisons (Saline vs. Small, Saline vs. Large, Small vs. Large) (see Figure 4-2 and 4-3). Using the intersection of the three different normalization methods, we found 28 genes to be different between saline and small suppressors, 21 genes between saline and large suppressors, and 11 between large and small suppressors (Figure 4-2).

**qRT-PCR Confirmation of RNA-Seq Differentially Expressed Genes**

Of the 60 genes found to be differentially expressed in the RNA-Seq experiment, we chose 10 genes to assay via qRT-PCR based on their relevance to brain and behavior function. From the qRT-PCR experiment, 6 genes were confirmed to be differentially expressed: Adora2a, Arhgef28, Bche, Fxyd6, Grap, and Pcdhga5 (Figure 4-3). The genes Pou3fa, Rims3, Sema3f and Zfp775 neither demonstrated differential expression, nor did they follow the same pattern of expression observed in the RNA-Seq experiment (Figure 4-4). This result was unexpected, and may have been caused by the use of the opposite hemisphere for qRT-PCR than the RNA-Seq analysis.

**Genomic Repeat Element Methylation**

One-way ANOVA was utilized to assess genomic repeat element methylation levels between groups. No differences in LINE-1 or ID methylation were detected (Figure 4-5).

**BiSulfite Amplicon Sequencing**

Cytosine methylation in specific genes is currently ongoing in the PSU College of Medicine Genome Sciences Core.
Table 4-1. Genes chosen for qRT-PCR confirmation

Statistically significant gene expression changes (with a >1.25-fold absolute difference) were chosen for further analysis based on relationship to brain. Genes for further BSAS analysis were chosen based on positive confirmation by qRT-PCR and the presence of CpG sites in gene regulatory regions. * denotes genes chosen for BSAS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Taqman primer/probe set</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arhgef28*</td>
<td>Rn01489752_m1</td>
<td>Axonal branching and synapse formation</td>
</tr>
<tr>
<td>Bche*</td>
<td>Rn00576087_m1</td>
<td>Pseudocholinesterase, associated with Alzheimer’s disease</td>
</tr>
<tr>
<td>Fxyd6*</td>
<td>Rn01421948_m1</td>
<td>Associated with psychotic disorders and schizophrenia</td>
</tr>
<tr>
<td>Pcdhga5*</td>
<td>Rn01405624_m1</td>
<td>Establishment of cell:cell connections in brain</td>
</tr>
<tr>
<td>Sema3f</td>
<td>Rn01447748_m1</td>
<td>Axon guidance during development, pro-apoptotic</td>
</tr>
<tr>
<td>Grap*</td>
<td>Rn01773048_m1</td>
<td>Intracellular trafficking, control of SNARE complex</td>
</tr>
<tr>
<td>Pou3f4</td>
<td>Rn00568456_s1</td>
<td>Mediates epigenetic signals associated with striatal development</td>
</tr>
<tr>
<td>Adora2a*</td>
<td>Rn00583935_m1</td>
<td>Adenosine receptor</td>
</tr>
<tr>
<td>Rims3</td>
<td>Rn00576000_m1</td>
<td>Regulates synaptic membrane exocytosis</td>
</tr>
<tr>
<td>Zfp775</td>
<td>Rn01763075_m1</td>
<td>DNA transcription, visual system</td>
</tr>
</tbody>
</table>
Figure 4-1. – A subset of rats suppresses saccharin intake when it is paired with the opportunity to self-administer heroin.

Rats were given the opportunity to ingest saline 5 minutes prior to a 6 hour heroin self-administration session (0.06 mg/0.2ml/infusion). A. Small suppressors increase intake of saccharin over trials, whereas large suppressors suppress saccharin. B. Heroin intake increases over trials in large suppressor group, whereas small suppressors do not escalate heroin intake. Saline n=5, small suppressor n=4, large suppressor n=5. Planned t-tests were conducted between small suppressors and large suppressors, *p<0.05, **p<0.01, ***p<0.001
Figure 4-2. – Representation of 3 different normalization methods used in the RNA-Seq analysis

- **Reads per kilobase per million mapped**
  - Normalizes by taking number of reads of a transcript and dividing by number of reads in experiment AND length of transcript (i.e. normalizes to gene size)

- **Differential Expression seq**
  - Normalizes to geometric mean of each transcript and normalizes to library size
  - $\sqrt{(8 + 2)} = 4 vs (8*2)/2 = 8$

- **Trimmed means of M-values**
  - Normalizes to the median amount of RNA of a set of samples (i.e. some samples may have more RNA than others, thus genes from a sample of small output may be under represented)

Figure 4-3. – Three normalization methods in the RNA-Seq experiment were used to select genes for further analysis.

- **Saline vs Large Suppressor**
  - 22
  - 12
  - 1
  - 9
  - 28
  - 6
  - 31

- **Saline vs Small Suppressor**
  - 129
  - 2
  - 4
  - 72
  - 21
  - 2
  - 98

- **Small vs Large Suppressor**
  - 27
  - 40
  - 14
  - 8
  - 11
  - 1
  - 56
Figure 4. QRT-PCR confirmations of differential gene expression changes in the RNA-Seq experiment.

Planned comparisons were employed to confirm gene-expression changes observed in the RNA-Seq experiment. * p<0.05, ** p<0.01. Saline in grey (n=5), Small suppressors (SS) in light blue (n=4), Large Suppressors (LS) in dark blue (n = 5). 60% of RNA-Seq illuminated differences were confirmed by orthogonal QRT-PCR.
Figure 4-5. QRT-PCR did not confirm some differential gene expression changes in RNA-Seq experiment.

Planned comparisons were employed in to confirm gene-expression changes observed in the RNA-Seq experiment. * p<0.05, ** p<0.01. Saline in grey (n=5), Small suppressors (SS) in light blue (n=4), Large Suppressors (LS) in dark blue (n = 5)
Figure 4-6. Genomic repeat methylation

Pyrosequencing of genomic repeat elements revealed no methylation differences between groups. One-Way ANOVA (n=2-5/group).
Primers for BSAS experiment were designed to target CpG islands on genes found to be differentially expressed in both RNA-Seq and QRT-PCR.
Table 4-2. BSAS Primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adora2a</strong></td>
<td>Adora2a-2F</td>
<td>5'-GGGAGGGGAGTATTTTGTTTATGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Adora2a-2R</td>
<td>5'-CTCTACCTACCCCTAAACCTTAACACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Adora2a-4F</td>
<td>5'-TGAGGAGTGAGGCCATAGGAGGAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Adora2a-4R</td>
<td>5'-CTCTCACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Adora2a-6F</td>
<td>5'-GGGAGGAGGAGTATTTTGTTGAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Adora2a-6R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td><strong>Argef28</strong></td>
<td>Argef28-1F</td>
<td>5'-TGAATATTGTTTATGTTTATGTTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Argef28-1R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Argef28-2F</td>
<td>5'-GGTAGATATTGTTTATGTTTATGTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Argef28-2R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td><strong>Bche</strong></td>
<td>Bche-1F</td>
<td>5'-GAATATAGTTGTTTATGTTTATGTTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Bche-1R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Bche-2F</td>
<td>5'-GAATATAGTTGTTTATGTTTATGTTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Bche-2R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Bche-3F</td>
<td>5'-GAATATAGTTGTTTATGTTTATGTTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Bche-3R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td><strong>Fxyd6</strong></td>
<td>Fxyd6-2F</td>
<td>5'-TTAAGGGGGGAAAAATGAAAGT-3'</td>
</tr>
<tr>
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<td>Fxyd6-2R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
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<td>Fxyd6-3F</td>
<td>5'-GGGGAAAAATGAAAGTAAAGT-3'</td>
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<tr>
<td></td>
<td>Fxyd6-3R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Fxyd6-4F</td>
<td>5'-AGTGGGTTGTTGTTTATGTTTATGTTTATGTTATG-3'</td>
</tr>
<tr>
<td></td>
<td>Fxyd6-4R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Fxyd6-5F</td>
<td>5'-GGAGTTTATGAAAGAAGGATGG-3'</td>
</tr>
<tr>
<td></td>
<td>Fxyd6-5R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td><strong>Pcdhga5</strong></td>
<td>Pcdhga5-1F</td>
<td>5'-GTTTTAAATGGAAGGTATTTTTGTTTATGTTTATGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Pcdhga5-1R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
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<td></td>
<td>Pcdhga5-2F</td>
<td>5'-GATTGGTTGTTGTTTATGTTTATGTTTATGTTG-3'</td>
</tr>
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<td></td>
<td>Pcdhga5-2R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
<tr>
<td></td>
<td>Pcdhga5-5F</td>
<td>5'-GTTAAATGGAAGGTATTTTTGTTTATGTTGTTTATGTTG-3'</td>
</tr>
<tr>
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<td>Pcdhga5-5R</td>
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<td>Pcdhga5-7F</td>
<td>5'-GAGGTTTGTATAGAAAGGTTTATGTTTATGTTGTTTATGTTG-3'</td>
</tr>
<tr>
<td></td>
<td>Pcdhga5-7R</td>
<td>5'-CTCTACCTACCTAAACCTTAAACCTTAC-3'</td>
</tr>
</tbody>
</table>
Discussion

Drug self-administration, in humans and animals, involves a complex orchestration of behaviors (e.g., drug seeking and taking, habit formation, responses to internal and external cues, etc.) and internal states (e.g., wanting and liking, attention, motivation, craving, withdrawal etc.). At the intersection of various limbic inputs, the mPFC is most recognized for its role in consolidating and evaluating received information and generating the most appropriate attentional and executive response. Accordingly, it is not surprising that addicted individuals demonstrate alterations in mPFC function (Forbes et al., 2014; Perry et al., 2011). Therefore, in order to investigate the question as to why some individuals succumb to drug addiction, while others remain resilient, the mPFC is a natural place to start.

In the present study, we examined the mPFC transcriptome of rats with different preferences for heroin and a heroin-associated taste cue. In short, when presented with an opportunity to ingest saccharin, followed by the opportunity to self-administer heroin, an outbred group of Sprague-Dawley rats will parse into two distinct phenotypes: those that demonstrate a liking of saccharin and have low/moderate interest in heroin (small saccharin suppressors) and those that suppress the intake of saccharin, and show a preference for heroin (large suppressors). Large suppressors demonstrate greater addiction-like behavior including: greater drug-loading behavior, shorter latency to first infusion, greater motivation to self-administer, greater drug seeking, and greater response to a drug-prime (Imperio et al., 2015).

One unexpected finding of this study was the occurrence of several failed qRT-PCR confirmation experiments. As reported in our RNA-Seq study of the NAc of LS and SS rats, genes found to be differentially expressed using the RPKM normalization method were positively confirmed by qRT-PCR when they were also found to be differentially expressed using the two other normalization methods: TMM and DESeq (Imperio et al., 2016). Therefore, in using 3
different normalization methods on our RNA-Seq data, we felt certain that we would uncover a list of genes that would be confirmed as changed by qRT-PCR. Unfortunately, due to sample restraints, we were unable to use the same RNA for qRT-PCR that we used for the RNA-Seq experiment. Instead, we isolated RNA from the opposite mPFC hemisphere of the same animals. Upon sacrifice, the two mPFC hemispheres were placed into separate tubes with the intention of using the tissues for a variety of assays (e.g., RNA-Seq, qRT-PCR, protein analysis), assuming that the hemispheres contained comparable molecular profiles. This aspect of our results and the findings of others (see “Limitations”—this Chapter and (Lee et al., 2015; Sullivan et al., 1994, 1995) led us to the hypothesis that the left and right mPFC may have different basal transcriptomes (see Chapter 5) and may respond differently to behavioral and pharmacological paradigms (see Chapter 3). Of note, although (Imperio et al., 2016) also used a presumably mixed set of left and right NAc for their study, qRT-PCR confirmations were performed with the same RNA as the RNAseq experiment, adding further support to our hemispheric differential gene-expression hypothesis.

**Differentially Expressed Genes**

Among the genes that were identified as differentially expressed between our groups, several stood out due to their influence on and/or association with brain function. Adora2a and FXYD6 (Chang et al., 2015; Choudhury et al., 2007) are two genes that are associated with schizophrenia. This is an interesting finding as schizophrenia is, at least in part, a disease characterized by a hyper-dopaminergic state and drugs of abuse increase DA signaling throughout the brain. FXYD6 is a gene that encodes the protein phosphohippolin, which is a regulator of NA⁺, K⁺-ATPase, a solute pump. FXDY proteins serve as an auxiliary subunit of NA⁺, K⁺–ATPases and modify the affinity of the pump for Na⁺ and K⁺ according to the needs of the cell. Thus, they serve as important regulators of cation balance and neuronal signaling (Mishra et al., 2011). In the present study, we
found FXYD6 to be decreased in a stepwise fashion in small and large suppressors, according to the amount of heroin taken. This indicates that the expression of this gene is responsive to the pharmacological environment and may mediate aspects of drug-associated changes in neuronal signaling.

Adora2a encodes the adenosine 2A receptor (A2A R). A2ARs are receptors coupled to Gs (van Aerde et al., 2015) and are found densely in striatal regions, but also are present in the prefrontal cortex. Activation of A2ARs in mouse prefrontal cortex brings about increased wakefulness, EEG activation and acetylcholine (ACh) release (Van Dort et al., 2009). As ACh enhances attention and arousal (Bloem et al., 2014) indirect activation of acetylcholine receptors in the mPFC, via increased A2AR signaling, may mediate the increased attention to drug-associated stimuli that is a hallmark of addiction. Activated A2ARs have been found to inhibit DA signaling in that they form heterodimers with dopamine D2 receptors (D2R), a relationship which acts as a D2R antagonist (Ferre, 1997). Of interest to our drug abuse studies, infusion of morphine into the brain (pontine reticular formation and substantia innominate) causes decreases in adenosine levels (Nelson et al., 2009). In our study, we found the Adora2a gene to be increased in response to the self-administration, regardless of dose. It is possible that decreased adenosine levels, brought about by opiate exposure, causes a compensatory increase in adenosine receptor levels, thereby indirectly increasing D2R availability. This could serve as a mechanism that enhances neuronal signaling in response to opiate-taking, and may expedite drug-induced receptor desensitization. Future studies should examine the impact of blockade or A2ARs on opiate SA and addiction-like behaviors.

Another set of genes found to be differentially expressed between the 3 experimental groups, Argef28, Grap, Pcdhga5, Rims3 and Sema3f, are involved in synaptic plasticity and neuronal signaling. Argef28 encodes a protein in the Rho guanine nucleotide exchange factor family. Members of this family activate GTPases by catalyzing the dissociation of the (inactivating) GDP so that a (activating) GTP can bind. Thus, they are regulators of G-protein
signaling. Grap, GRB2-related adapter protein, is a member of the growth factor receptor bound protein family. Members of this family bind to activated receptors with phosphorylated tyrosines and activate the RAS signaling pathway (Trub et al, 1997). Sema3f encodes a member of the semaphorin III family, proteins that are involved in axon guidance during neuronal development (Luo et al, 1993). Of additional interest, Sema3f has been found to induce apoptosis and inhibit cell proliferation in endothelial cells (Nasarre et al, 2003). Pcdhga5 is a member of the protocadherin gamma family. These are calcium-dependent cell-cell adhesion molecules. The protocadherins are cell surface proteins found on dendrites, axons and at synapses and are highly expressed in the developing CNS (Weiner and Jontes, 2013). In the cerebral cortex, selective loss of the protocadherin gamma proteins results in a reduction of dendritic arborization of pyramidal neurons (Garrett et al, 2012). Interestingly, in our study, drug-taking resulted in decreased expression of Pcdhga5, again, in a stepwise manner, with the large suppressor group having the lowest levels of expression. Chronic administration of morphine (Robinson and Kolb, 1999) and cocaine (Rasakham et al, 2014) is associated with decreased dendritic branching of mPFC pyramidal neurons. Decreased expression of Pcdhga5 could mediate, in part, this change in mPFC structure and function.

Gene Expression Patterns

Much like our RNA-Seq experiment within the NAc (Imperio et al, 2016), we observed several different patterns of gene expression in the present study. Gene expression patterns between the three experimental groups can inform us as to the possible role or responsiveness of a given gene to pharmacological and/or behavioral influence. For example, there are several genes in our study that reflect a dose or drug effect. That is, gene expression increases or decreases in a stepwise pattern according to how much drug was taken (saline = none, SS = small/moderate, LS = large).
This can be seen in genes such as Arhgef28, Fxyd6 and Pcdhga5. There are other genes that increase or decrease in response to receiving any amount of drug. Examples of this are: Adora2a, Behe, Sema3f and Zfp775, which show no difference between the two heroin-taking groups, but are both different than saline. There are also a small number of genes that are differentially expressed between the most behaviorally different of the experimental groups: SS and LS. Genes in this group include Grap, Pou3fa and Rims3. In this last, and rarer (as discussed below), group of genes, the gene expression of saline animals is at a midpoint between SS and LS. As we have discussed previously (McFalls et al, 2016), presumably, our saline group is a mix of SS and LS that have not been behaviorally stratified by our model. Therefore, the genes in this last group may be particularly responsive to, or perhaps, precede behavioral differences, and may be genes that confer resilience or vulnerability to an individual.

The number of genes differentially expressed between our three experimental groups, as well as magnitude of change, reflects the tight regulation of gene expression in the brain. For example, the greatest number of genes differentially expressed occurred between saline animals and LS; the most pharmacologically different group (no drug intake vs. large heroin takers). It is likely that a strong pharmacological agent, such as heroin, would have a more profound effect on brain chemistry than behavior. Accordingly, the second largest group of differentially expressed genes occurred between saline and SS, again, pointing toward a drug effect. The smallest number of genes changed occurred between the two groups that were behaviorally the most different: SS and LS.

**CRF Pathway Gene Expression**

We have previously reported differential expression of CRFR1 and GluR1 in the mPFC of LS and SS rats. In the current study, those genes were not called as differentially expressed using the 3
normalization methods. There are several reasons that could account for this. First, the rats chosen for this study, while similar, were not the exact group of rats chosen for the study in McFalls et al., 2016 (88% overlap between LS and SS). Secondly, whereas in the CRF study, we used a hypothesis driven t-test, in the present study, an ANOVA was used to assess differences between the three groups (saline, LS and SS).

**Limitations**

A major limitation of this paper was the use of a mix of unknown right and left hemispheres for the RNA-Seq analysis, and a mix of opposite hemispheres for qRT-PCR confirmation. While it is customary to perform analysis on one side and confirmation or extension on the other (Kopec et al., 2017; Kremer et al., 2013), reports are emerging of laterality differences between the left and right mPFC. Of interest to the drug-abuse field, differences in DA projections have been found between the right and left mPFC (Sullivan et al., 1994, 1995) and Staiti et al. (2011) found increased extracellular DA on the right mPFC (Staiti et al., 2011). It is likely that these differences also extend to gene expression. As a strength, it could be deduced that the genes that were confirmed by QRT-PCR (with opposite hemispheres) are those that are uniformly changed (across hemispheres) in response to the pharmacological or behavioral manipulation in our model. Future studies should examine the laterality of these genes and others through the use of QRT-PCR or RNA-Seq.

**Conclusion**

In a rat model of reward devaluation and heroin self-administration, an outbred group of Sprague-Dawley rats will parse into two heroin-taking phenotypes: saccharin-accepting (small suppressor) and saccharin-suppressing (large suppressor). Using RNA-Seq, we analyzed the mPFC
transcriptome of rats of these divergent phenotypes as well as rats with no access to heroin (saline controls). Genes found to be differentially expressed include those associated with schizophrenia, signal transduction, development and synaptic plasticity. Additionally, analysis of gene expression patterns between groups revealed that some genes may be responsive to the dose of drug taken, while others increase or decrease regardless of dose. Others appear to be related to behavioral differences and may represent genes that confer vulnerability or resilience to addiction-like behavior. This study elucidates gene expression differences in the mPFC that may contribute to individual responses to drugs of abuse and provide new pharmacological targets for the treatment of drug-addiction.
Chapter 5
Differential Gene-Expression in the Left and Right Medial Prefrontal Cortex of Rats

McFalls AJ, Ballard MB, Hajnal A, Freeman WM, Grigson PS,
Vrana KE and Imamura Y

Abstract

The medial prefrontal cortex (mPFC) is a recently evolved structure whose main functions involve integrating and analyzing signals from various limbic structures and using that information to alter decision-making and executive function. Accordingly, the mPFC is also involved in orchestrating the neural and humoral stress response through connections with the amygdala and hypothalamic pituitary adrenal axis (HPA axis). It is a common laboratory practice to utilize one hemisphere for one kind of molecular analysis (e.g., protein expression) and to perform a different kind of analysis on the other (e.g., mRNA expression). However, convenient this approach, reports are emerging of lateral asymmetries in the mPFC (of rats and humans) in areas such as structural morphology, connectivity, and neurotransmission. Therefore, the aim of this study was to examine the mPFC transcriptome of treatment naïve animals in order to uncover any left/right differences that may exist. Five untreated male Sprague-Dawley rats were sacrificed and the mPFC (containing

4 This Chapter is comprised of material from a manuscript that will be submitted to Neuroscience. The experiments were designed and executed by AJM (in consultation with KEV and PSG). AJM and SMB handled the animals. AH performed subject sacrifice and designed and executed brain dissection (with the aid of AJM). All the molecular experiments were designed and executed by AJM (in consultation with KEV and YI). GB assisted with RNA extraction, cDNA synthesis and qRT-PCR. WMF and YI performed the differential RNA-Seq analysis. AJM prepared the manuscript and all Figures (with editorial assistance from AH, PSG, WMF, and KEV).
infralimbic and prelimbic corticies) was harvested from the left and right hemisphere. RNA-Seq analysis was conducted on the left and right mPFC and 487 genes were found to be differentially expressed between the hemispheres. Ingenuity Pathway Analysis revealed that many of the genes were involved in pathways such as cell signaling and cell morphology. These experiments add to the growing body of literature of hemisphere asymmetries in the mPFC and provide evidence that consideration should be given to hemisphere when conducting molecular experiments on the mPFC.
Introduction

The medial prefrontal cortex is a recently evolved structure most known for its role in attention and decision making. The mPFC receives various inputs from structures of the limbic system and coordinates the most appropriate response to a given stimulus. Accordingly, the rat mPFC has been implicated in several higher order functions such as learning, memory, stimulus association, temporal sequence of events, decision-making and goal-directed behavior and is also an integral part of mediating the stress response.

The mPFC participates in both turning on and turning off the stress response; an effect that has been shown to be dependent on hemisphere specificity. The mPFC expresses abundant levels of glucocorticoid receptors and thus participates in direct negative regulation of the HPA axis. Under situations of stress, right mPFC neurons function in an “all or none” fashion, mediating the immediate hormonal response to stress (Hewig et al, 2008; Kalin et al, 1998). In fact, lesions of the right mPFC have been shown to increase stress resilience, a response attributed to decreased stress hormone production (Sullivan and Gratton, 1999). Conversely, left mPFC neurons respond to stress in a graduated manner, activity being linked to stress severity (Lee et al, 2015) and likely mediate an adaptive response to chronic stress. Resilience or vulnerability to chronic stress has been linked to individual differences in left/right mPFC dominance. Mice behaviorally phenotyped as stress-susceptible in a social defeat paradigm show decreased activity in the left mPFC, while phenotypically resilient mice show normal firing in the left mPFC. Moreover, photoactivation of neurons in the left mPFC of stress-susceptible mice reversed social-avoidant behavior. In humans, greater activity in the right mPFC is associated with anxiety (Wiedemann et al, 1999), depression (Field et al, 2000), and greater negative response to negative stimuli (Davidson et al, 2000; Jackson et al, 2003; Sutton and Davidson, 2000). Conversely, individuals with greater activity in the left
mPFC demonstrate inhibition of negative emotion and a greater positive response to positive stimuli (Jackson et al, 2003; Sutton et al, 2000).

The right and left mPFC also demonstrate structural asymmetries. In unstressed animals, the left mPFC has higher levels of gliogenesis than the right mPFC. However, under conditions of chronic stress, the left mPFC undergoes structural changes and volume loss (Drevets et al, 1997) and higher levels of cell proliferation and survival is observed in the right mPFC (Czeh et al, 2007). Moreover, several laboratories have reported hemisphere differences in regards to dopamine projections from the VTA to the mPFC (Sullivan et al, 1994, 1995). Staiti and colleagues (2011) found increased basal levels of extracellular dopamine and norepinephrine in the right mPFC (Staiti et al, 2011).

It is well known that early life experiences can greatly augment an organism’s response to stressful stimuli, and the ability to handle stress well or succumb to anxiety can be linked to a differential response of the right and left mPFC. In an experiment investigating early life experiences on mPFC function, Stevenson et al. (2008) found that rats exposed to maternal separation (a stressful experience that has been shown to impact later functions such as memory, stress responsivity and drug-taking behavior) (Stevenson et al, 2008) had lower basal right mPFC activity than rats that were handled (a procedure shown to increase stress resilience (Meaney et al, 1996)). Further, after administration of a GABA partial inverse agonist (FG-7412) (shown to cause anxiety-like behavior) rats that had experienced maternal separation showed increased firing in the left mPFC, and decreased firing in the right mPFC (Stevenson et al, 2008). As the development of several stress-related signaling mechanisms occurs early in life, including maturation of mPFC GABA neurons, establishment of mesocortical DA projections and glucocorticoid receptor expression, it is likely that early life experience causes further asymmetrical mPFC development.

Despite these emerging reports of lateral differences in connectivity, neurochemistry and behavioral functions of the mPFC, it remains standard practice to utilize one hemisphere for an
experimental protocol and assume the region is the same on the other side (i.e., perform analysis on one side and confirmation or extension on the other (Kopec et al, 2017; Kremer et al, 2013)). Indeed, our own laboratory recently unintentionally discovered that gene expression in one hemisphere of the mPFC does not necessarily reflect gene expression in the opposite hemisphere (see Chapter 4). Therefore, the aim of this study was to discover if and what genes are expressed differently in the left and right mPFC of the Sprague-Dawley rat (a general purpose and widely used laboratory animal), so that we and future researchers will give consideration to hemisphere differences when examining neuromolecular phenomena.
Materials and Methods

Subjects

Eight male Sprague-Dawley rats, obtained from Charles River at approximately 90 days of age were the subjects of this study. Rats were individually housed in stainless steel suspended cages in a humidity-controlled environment and maintained on a 12/12 h light dark cycle. All rats were handled, weighed daily and food and water was available ad libitum. Following one week of quarantine, all rats were handled every morning for 7 days and they weighed approximately 300-350 grams at the time of sacrifice.

Sacrifice and Dissection

Sacrifice was performed by first anesthetizing rats with isoflurane, followed by rapid decapitation. Brains were immediately removed, placed into ice cold phosphate buffered saline, dissected and then flash frozen for molecular analysis. The following brain region was harvested as described previously (Heffner et al, 1980): mPFC, containing the prelimbic and infralimbic cortex, (AP: +3.72 to +2.76), from Bregma based on (Paxinos and Watson, 6th Edition, 2007)). The left and right hemispheres were collected into separate, labeled tubes, and the correct hemisphere was confirmed by both the dissector and the person collecting the dissected region.

Subject Selection

Five out of eight rats were chosen for the RNA-Seq analysis based on the following criteria: 1) both mPFC hemispheres were within close proximity to the 22mg average mPFC weight (ensuring approximately correct tissue size); 2) smallest differences in weight between the left and right
mPFC (ensuring equal amounts of tissue taken); and 3) RNA integrity number (RIN) was 
(following RNA isolation) >7.5 (the RIN is based on the ratio of 28S to 18S rRNA in an 
electropherogram trace. Generally, an RIN of >7 is considered good quality RNA).

**RNA Isolation and cDNA Synthesis**

DNA and RNA was isolated from mPFC tissue using the Allprep DNA/RNA isolation kit (Qiagen, 
Irvine, CA) and complimentary DNA (cDNA) was generated from 1 ug RNA with the ABI High 
Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). RNA quality and 
quantity was assessed using the RNA 6000 Nano Assay with an Agilent 2100 Bioanalyzer (Agilent, 
Palo Alto, CA).

**Library Construction and Next Generation Sequencing**

Libraries were constructed using the Illumina Truseq Stranded HT library that retains the 
directionality of transcripts (Illumina, San Diego, CA). Polyadenylated tails of mRNA were 
captured via oligo-dT magnetic beads and then purified. mRNA was then chemically-fragmented 
upon elution and cDNA was synthesized. In order to maintain directionality of the transcripts, 
dUTP (instead of dTTP) is incorporated into the second strand during cDNA synthesis; the 
polymerase is unable to read past this nucleotide. After synthesis, cDNA products underwent end 
repair, the addition of a single adenosine base, and ligation of the adapter sequences. cDNA 
products were further purified for sequencing and enriched via PCR. Libraries were quantified by 
qRT-PCR (Kapa Biosystems) and sized by TapeStation (Agilent). The cDNA libraries were then 
sequenced on an Illumina Hiseq 2500 (2x100bp).
RNA-Seq Data Analysis

Analysis of the RNA-Seq data for differential expression was conducted concurrently using two different algorithms and normalization methods and produced very similar results.

Strand NGS Analysis and RPKM:

After sequencing, reads were aligned, filtered, trimmed and analyzed for differential expression using the strand NGS software package (Agilent). Reads were aligned to the Rnor 6 build of the rat genome. Alignment and filtering included the following read quality metrics: 2 bp were trimmed off of the 3’ and 5’ end of each read, low quality reads were trimmed off of the 3’ end (Q<20), reads that were duplicates, ambiguous or in the wrong direction were filtered out, and only reads with an 80% minimum match length with the reference sequence were included. Reads were aligned in a directional manner with read 1 in the reverse direction, and read 2 in the forward direction. Normalization was performed using Reads per Kilobase per Million Mapped (RPKM). Paired t-test (α<0.05) was utilized to discover differentially expressed genes between right and left hemisphere. A 1.5-fold cutoff was further employed to isolate genes of physiological relevance (not just statistical significance).

R Analysis and FPKM:

A bowtie2 index was built for the Rattus_norvegicus.Rnor_6.0 genome assembly using bowtie version 2.1.0. The RNA-seq reads of each of the 10 samples were mapped using Tophat version 2.0.13 (Trapnell et al, 2009) supplied by Ensembl annotation file; Rnor_6.0.87.gtf. Each sample's raw read counts were quantified using using HTSeq version 0.6.1 (Andres et al., 2015) supplied by Rnor_6.0.87.gtf. Differential gene expression analysis was carried out using edgeR in a paired
design (Robinson et al., 2010). Significance was determined with a False Discovery Rate (FDR) adjusted p < 0.05 using the Benjamini-Hochberg method.

**Gene Selection Criteria**

Genes selected for further qRT-PCR confirmation were those that were found to be differentially expressed in the RNA-Seq analysis (statistically significant plus ≥1.5 fold difference) and whose function had known ties to brain function and/or behavior (see Table 1). Further, we preferentially chose genes that had larger fold changes, as these genes were more likely to play a significant role in functional differences (than those with smaller fold changes).

**Confirmatory qRT-PCR**

Genes found to be differentially expressed by RNA-Seq were assessed by the orthogonal method, RT-qRT-PCR using TaqMan assay on demand primer/probe sets (Life Technologies, Carlsbad, CA) (Table 1) and a QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems) according to our standard methods (Bixler et al., 2011; Masser et al., 2014). Relative gene expression was calculated with Expression suite v 1.0.3 software using the 2−ΔΔCt analysis method with β-actin as an endogenous control. qRT-PCR confirmation analysis was performed in the same subjects used in the RNA-Seq analysis (n=5/group). Independent t-tests were used to compare results from qRT-PCR analysis based on prior differences found using RNA-Seq.
**Ingenuity Pathway Analysis**

Analysis of diseases and functions, signaling pathways, and upstream regulators was conducted using Ingenuity Pathway Analysis (IPA, www.qiagen.com/ingenuity, San Diego, CA).
Results

RNA-Seq Analysis
Differentially expressed transcripts between groups were identified though a pairwise design ($\alpha<0.05$). These transcripts were further filtered to include only those that had a $>|1.25|$ fold difference between the hemispheres in order to uncover genes of physiological relevance (not just statistical significance). This resulted in a total of 487 differentially expressed transcripts between the left and right mPFC of the 18,318 transcripts with rat RefSeq annotations that were called as expressed in the mPFC; this represented transcripts from 2.6% of observed genes. Differentially expressed transcripts were normalized using the RPKM and FPKM method (Figure 5-1). Depth of coverage in the RNA-Seq experiment was 100x. Both RPKM analysis and FPKM analysis showed that the majority of differentially expressed genes were down-regulated on the left mPFC (464) while only a small number of genes were down-regulated on the right (23). While our first thought was that this was a sequencing error, orthogonal qRT-PCR confirmed the differences observed in the RNA-Seq experiment (see “qRT-PCR Confirmation of RNA-Seq Differentially Expressed Genes”).

qRT-PCR Confirmation of RNA-Seq Differentially Expressed Genes
Of the 487 genes found to be differentially expressed in the RNA-Seq experiment, we chose 12 genes to assay via qRT-PCR based on their relevance to brain function and behavior (Table 5-1). Consideration was also given to genes that demonstrated high expression (as exemplified by read count) as those would likely be physiologically relevant. All 12 genes confirmed the differential expression observed in the RNA-Seq experiment (Figure 5-2). We also observed that, regardless of magnitude, in the 12 genes chosen, gene expression was always higher on the right side, when comparing the left and right hemisphere gene expression from the same animal (see Figure 5-3).
Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) was conducted on the genes found to be differentially expressed in order to identify networks, signaling pathways and functions affected by our list of genes. The top brain-related networks identified by the IPA analysis included cell-to-cell signaling and interaction, cellular development, cell morphology, cellular growth and proliferation (Figures 5-3 and 5-4), as well as cell morphology and cellular assembly, organization, function and maintenance (Figure 5-5)

Table 5-1. Genes chosen for qRT-PCR confirmation
Genes from the RNAseq experiment were chosen based on relationship to brain.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>TaqMan primer/probe set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrnb3</td>
<td>Nicotinic acetylcholine subunit beta 3</td>
<td>Rn00592317_m1</td>
</tr>
<tr>
<td>Igf2</td>
<td>Insulin-like growth factor</td>
<td>Rn01454518_m1</td>
</tr>
<tr>
<td>Igfbp2</td>
<td>insulin like growth factor binding protein</td>
<td>Rn00565473_m1</td>
</tr>
<tr>
<td>Nurp1</td>
<td>Nuclear protein 1</td>
<td>Rn00586046_m1</td>
</tr>
<tr>
<td>Ptgds</td>
<td>Prostaglandin D2 synthase</td>
<td>Rn00564605_m1</td>
</tr>
<tr>
<td>Ptgfr</td>
<td>Prostaglandin F receptor</td>
<td>Rn00565423_m1</td>
</tr>
<tr>
<td>Slc13a4</td>
<td>Solute Carrier Family 13 (Sodium/Sulphate Symporters), Member 4</td>
<td>Rn01747911_m1</td>
</tr>
<tr>
<td>Slc6a13</td>
<td>Solute Carrier Family 6 (Neurotransmitter Transporter, GABA), Member 13</td>
<td>Rn01492499_m1</td>
</tr>
<tr>
<td>Slc6a20</td>
<td>Solute Carrier Family 6 (Proline Imino Transporter), Member 20</td>
<td>Rn00591045_m1</td>
</tr>
<tr>
<td>Sned1</td>
<td>Sushi, nidogen and EGF-like domains 1</td>
<td>Rn01427574_m1</td>
</tr>
<tr>
<td>Thbs2</td>
<td>Thrombospondin2</td>
<td>Rn02111874_s1</td>
</tr>
<tr>
<td>Trpv1</td>
<td>Transient receptor potential cation channel subfamily V member 4</td>
<td>Rn0143037_m1</td>
</tr>
</tbody>
</table>
Figure 5-1. – Differentially expressed genes between left and right hemisphere.

A. Heatmap of 487 differentially expressed transcripts between left and right mPFC. Red=left mPFC, Olive=right mPFC. B. RPKM normalization revealed 464 genes to have lower expression on the left and 23 genes to have lower expression on the right out of the 18,318 transcripts called as expressed in the mPFC (n=5/side) (α<0.05). Transcripts were filtered to include only those that had a >|1.25| fold difference between the hemispheres in order to focus on potential genes of physiological relevance.
Figure 5.2. – qRT-PCR confirmation of differentially expressed genes between left and right hemisphere.

Planned t-tests were utilized to confirm differential expression of genes changed in the RNA-seq experiment (n=5/side). *p<0.05, **p<0.01
Figure 5-3. All genes chosen for confirmatory qRT-PCR demonstrate higher expression in the right hemisphere compared to the left.

Gene expression data obtained from the qRT-PCR experiments (n=5/side).
IPA analysis of differentially expressed genes revealed that 16 genes are involved in cell-cell signaling, cellular development, growth and proliferation (n=5/side). Green-shaded molecules are those found to be more highly expressed in the right hemisphere in the RNA-Seq analysis, red-shaded molecule are those found to be more highly expressed in the left hemisphere. Genes in this network are involved in the uptake of nutrient for cellular energy, calcium signaling and structural protein synthesis.
IPA analysis of differentially expressed genes revealed that 16 genes are involved in cell-cell signaling, cellular development, growth and proliferation (n=5/side). Green-shaded molecules are those found to be more highly expressed in the right hemisphere in the RNA-Seq analysis, red-shaded molecules are those found to be more highly expressed in the left hemisphere. Genes in this network include structural proteins, nuclear proteins that influence cell-type differentiation and neuroprotection.
Figure 5-6. Gene network involved in cell morphology and cellular assembly, organization, function and maintenance

IPA analysis of differentially expressed genes revealed that 25 genes are involved in cell morphology and cellular assembly, organization, function and maintenance (n=5/side). Green-shaded molecules are those found to be more highly expressed in the right hemisphere in the RNA-Seq analysis. Genes in this network include those involved in adult neural stem cell self-renewal and remodeling and formation of the extracellular matrix.
**Discussion**

Given the known structural and signaling differences between the left and right mPFC, the current study examined whether the mPFC transcriptome would also demonstrate lateral differences. Here, we show that in a treatment *naïve* set of Sprague-Dawley rats, 487 genes are differentially expressed between the right and left mPFC. Genes found to be differentially expressed are involved in cell signaling and cell growth, development and proliferation and cellular assembly and maintenance.

The existence of differentially expressed genes between the left and right mPFC is not surprising given the numerous accounts of lateralization in behavior (Sullivan, 2004), emotion and cognition (Sutton *et al.*, 2000), control of the HPA axis (Sullivan *et al.*, 1999), as well as signaling (Staiti *et al.*, 2011; Stevenson *et al.*, 2008). The left and right mPFC have different structural responses to stress. Czeh and colleagues have observed that the left mPFC of unstressed animals has higher levels of gliogenesis than the right mPFC. However, higher levels of cell proliferation and survival is observed in the *right* mPFC after chronic stress, whereas the left mPFC demonstrates greater structural and volume loss (Czeh *et al.*, 2007). This occurrence appears to be applicable to humans. Mothers that are under high levels of stress show decreased activity of the left mPFC and predominate activity in the right mPFC, while mothers under low levels of stress demonstrate a predominance of left mPFC activity (Chen *et al.*, 2015). As several laboratories have reported hemisphere differences in neurotransmitters such as dopamine and norepinephrine (Sullivan *et al.*, 1994, 1995) (neurotransmitters that both respond to stressful stimuli and regulate synaptic plasticity (Gurden *et al.*, 2000; Otani *et al.*, 1998)), it was gratifying to see that several of the genes found to be differentially expressed in our experiments are involved in networks that mediate cell signaling and morphology. It is possible that many of the genes uncovered in this experiment are responsible, in part, for the structural differences and responses between the left and right mPFC. Future studies should examine the responsiveness of these genes in different stress and behavior paradigms.
Acetylcholine Signaling in the mPFC

One of the genes with the greatest difference in expression between the left and right mPFC was Chrnb3. Chrnb3 encodes a beta subunit of the nicotinic acetylcholine receptor, a ligand gated cation channel permeable to Na⁺, K⁺ and Ca²⁺ (Beker et al., 2003). Chrnb3 is most known for its association with nicotine dependence phenotypes in human populations (Rice et al., 2012). Agonism of the nicotinic acetylcholine receptor in the mPFC brings about improvements in working memory (Raybuck and Gould, 2010), as well as attention and executive functions (Bloem et al., 2014). Accordingly, microdialysis studies have shown that attentional tasks are accompanied by a rise in acetylcholine in the mPFC (Passetti et al., 2000). The mPFC receives dense cholinergic innervation from several brain regions including the basal forebrain (Eckenstein and Baughman, 1984), the pedunculopontine nucleus and the lateraldorsal tegmental area (Eckenstein et al., 1988), although cholinergic neurons are present in the PFC, as well. Layers I-III and layer V are the most densely innervated (Eckenstein et al., 1988). Of note, polymorphisms in the Chrnb3 gene are associated with dizziness upon the first inhalation of a cigarette – an occurrence that predicts later sustained smoking behavior (Bloem et al., 2014; Pedneault et al., 2014). Of note, this is the first report, to our knowledge, of differential Chrnb3 gene expression between mPFC hemispheres. Another gene observed to have increased expression in the right hemisphere was insulin like growth factor II (IGF2), which has been shown to potentiate acetylcholine release in the prefrontal cortex (Napoli et al., 2008). Taken together, our observation of increased Chrnb3 and IGF2 mRNA in the right mPFC may, in part, mediate the right mPFC-specific activation of the HPA axis. Future studies should examine the interplay of IGF2 and ACh signaling in an organism’s response to stress.
Insulin-Like Growth Factor Family and Depression

The occurrence of differential expression of IGF2 and IGFBP2 is an interesting finding given the known role of insulin signaling in mood regulation and depression and the hemisphere-specific control of stress responsivity. The IGFs (IGF2 as well as IGF1) are neurotrophic factors shown to regulate synaptic plasticity and adult neurogenesis and bind to the insulin-like growth factor receptor 1 and the insulin receptor (both receptor tyrosine kinases). IGFBP2 is the predominant binding partner of the IGFs and has been shown to both attenuate and strengthen IGF signaling in a tissue-specific manner (Firth and Baxter, 2002; Mohan and Baylink, 2002). Insulin resistance and diabetes are associated with greater likelihood of depression (Musselman et al, 2003; Rasgon et al, 2003; Timonen et al, 2005). Moreover, improvements in depression symptoms is associated with increased insulin sensitivity (Hood et al, 2011), an effect that is linked to alterations in various members of the insulin-like growth factor family. Several laboratories have observed that administration of IGF1 produces decreases in depressive-like symptoms in animal models (using a variety of behavioral assays such as the forced swimming test and tail suspension) (Hoshaw et al, 2008; Hoshaw et al, 2005). Bezchlibnyk and associates observed that patients with untreated depression had decreased levels of IGFBP2 mRNA in the prefrontal cortex (Bezchlibnyk et al, 2007). In addition to modulating IGF signaling, IGFBP2 may reduce depressive like symptoms through IGF-independent mechanisms. Burgdorf, Colechio and colleagues found that administration of IGFBP2 attenuates PTSD-like symptoms in rats, and further, causes increases in dendritic spine density in the mPFC and dorsal hippocampus, an effect that is independent of IGF1 (Burgdorf et al, 2017). Further, rough and tumble play (an activity shown to increase resilience to stress in rats) causes the upregulation of IGFBP2 in the mPFC (Burgdorf et al, 2010). Although there are no prior reports of insulin signaling laterality, our observation of increased IGF signaling molecules in the right mPFC of untreated rats may, in part, underlie the mechanism by which right
mPFC dominance is associated with decreased stress resilience and depression. Future studies should examine hemisphere-specific IGF signaling in paradigms that examine stress resilience and sensitivity.

**Gene Comparison with Others**

Krishnan and colleagues performed a DNA microarray study to illuminate molecular adaptations and differences in the VTA and NAc of mice that were susceptible and unsusceptible to stress. Of interest, they found that mice of the unsusceptible phenotype had several K+ channels upregulated in the VTA. The authors hypothesized that this mechanism mitigated the strength of increased stress-induced DA transmission, thereby decreasing the deleterious effects of stress (Krishnan et al., 2007). We cross-referenced the list of differentially expressed genes published in Krishnan et al. with our own list of hemispherically differentially expressed genes and found several similarities. First, the right hemisphere had higher expression of 3 K+ channels: Kcnk5, Kcnk6, and Kcne4. Although we do not know in which cell type these genes were upregulated, it is possible that the rectifying properties of these K+ channels are the mechanism by which the right hemisphere has been observed to be structurally resistant to stress (Czeh et al., 2007; Drevets et al., 1997), particularly given the reports of laterality in DA transmission from the VTA to the mPFC (Sullivan et al., 1994, 1995). Krishnan et al. also found that phosphodiesterase 3a (Pde3a) is decreased in the unsusceptible group, while we also found it to be decreased in the left hemisphere.

**Limitations**

As the aim of this study was to uncover differentially expressed genes between the left and right hemisphere of naïve and untreated Sprague-Dawley rats, it remains unclear as to how gene
expression is lateralized following environmental factors such as stress (which has been shown to alter mPFC laterality), as well as behavior or drug administration. Future studies should examine left/right asymmetries in a number of commonly used laboratory techniques (e.g., drug administration, behavioral paradigms) to provide a more complete picture as to how to utilize the left and right mPFC in molecular analyses. Moreover, as our analysis featured only one age group (young adult), one species (Sprague-Dawley) and one sex (male), future studies should also examine hemisphere differences in other commonly used laboratory animals, commonly used age groups, and especially females across the estrus cycle. Another major limitation of our study was that we used mRNA expression as a surrogate for protein expression. Future studies should also examine the proteomic landscape of the left and right mPFC.

**Conclusions**

Here we show that an outbred, untreated group of male Sprague-Dawley rats demonstrates lateral differences in gene expression in the mPFC. Differentially expressed genes were identified to reside in networks such as cell signaling and cell morphology. These data add to the growing body of literature that suggests that the left and right mPFC are structurally and functionally different and provides further evidence that molecular studies on the mPFC should be conducted with regard to hemisphere.
Chapter 6

Exposure to Environmental Enrichment Attenuates Addiction-like Behavior and Alters Molecular Effects of Heroin Self-administration in Rats

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Kent. E. Vrana, Patricia S. Grigson, and Willard M. Freeman

Abstract

Environmental factors profoundly affect the addictive potential of drugs of abuse, and may also modulate the neuro-anatomical/neuro-chemical impacts of drug abuse and propensity to relapse. The present study examined the impact of environmental enrichment on heroin self-administration, drug-abuse related behaviors, and molecular processes proposed to underlie these behaviors. Male Sprague-Dawley rats in standard and enriched housing conditions intravenously self-administered heroin or saline for 14 days. Enrichment attenuated progressive ratio extinction, and reinstatement responding after 14 days of enforced abstinence. Molecular mechanisms, namely DNA methylation and gene expression, have been proposed to underlie abstinence-persistent behaviors. Global reductions in methylation have been reported to coincide with drug-abuse liability, but no differences in total genomic methylation or repeat element methylation were observed in CpG or

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5 This Chapter is comprised of material from a manuscript submitted to Neuropsychopharmacology. The initial behavioral heroin self-administration experiments were designed and executed by CGI and AC (in consultation with PSG). The molecular experiments were designed and executed by CGI, AJM, NH, DR and GB (in consultation with KEV, PSG and WMF). CGI wrote the first draft as a part of his thesis dissertation. AJM executed data analysis for behavior, gene expression and BSAS site-specific methylation experiments, and prepared the manuscript and Figures (with editorial assistance from WMF).
non-CpG (CH) contexts across the mesolimbic circuitry as assessed by multiple methods. mRNA expression associated with drug-seeking, taking and abstinence were examined. Immediate early gene expression was suppressed in mesolimbic regions in heroin-taking subjects in an enriched environment compared to all other groups. A site-specific methylation analysis of EGR1 and EGR2 using bisulfite amplicon sequencing (BSAS) revealed hyper-methylation of CpG sites in the proximal promoter region of EGR2 in the mPFC of standard saline animals that was associated with increased mRNA expression. Taken together, these findings illuminate the impact of drug taking and environment on the epigenome in a locus and gene-specific manner and highlight the need for positive, alternative rewards in the treatment and prevention of drug addiction.
Introduction

Heroin addiction is characterized by chronic relapse to abuse even after years of abstinence. Heroin use is currently surging, as abusers of prescription opioids turn to cheaper and more accessible heroin (Cicero et al, 2014; Diamond et al, 1972; Lankenau et al, 2012). Half of individuals engaging in heroin use will devolve into substance dependence (U.S. Dept of Health and Human Services, 2012), while less than half of heroin addicts will achieve long-term abstinence (>5 years) (Hser, 2007). Therefore, understanding the neurobiological mechanisms that drive addiction and relapse could aid in preventing heroin addiction and maintaining abstinence in the recovering abuser.

The Effect of Environment on Drug Self-Administration

Genetic and environmental factors both contribute to addiction susceptibility (Nielsen et al, 2012). This interplay of nature and nurture has long been studied, but their relationship in drug abuse processes remains unclear. While negative or aversive experiences can cause a predilection to substance abuse and relapse, positive experiences can be protective (Eitan et al, 2017; Stairs and Bardo, 2009). Environmental enrichment in the laboratory is normally considered a positive experience, consisting of engaging stimuli and social interactions (Diamond et al, 1972; van Praag et al, 2000). Exposure to enrichment during adolescence can decrease addiction-like behaviors to cocaine later in life (Solinas et al, 2008; Solinas et al, 2010). Moreover, environmental adulthood enrichment during enforced cocaine abstinence diminishes cue-induced relapse-like behaviors (Chauvet et al, 2009) and these effects disappear with removal of environmental enrichment (Nader et al, 2012). We have shown that an enriched environment (i.e., group housing and novel objects) in adulthood, before and during acquisition of cocaine self-administration, significantly diminished
acquisition of cocaine self-administration (Puhl et al., 2012). However, little is known about the effectiveness of enrichment on addiction-like behaviors with heroin self-administration. Recent reports suggest a positive impact of environmental enrichment on heroin-responsive behaviors, with decreased heroin reinstatement (Galaj et al., 2016) and increased abstinence in a conflict model (Peck et al., 2015). Given the ongoing opioid epidemic, modifying environmental factors and examining their impacts on opiate addiction is highly relevant. The present study examined the effects of environmental enrichment (i.e., group housing, novel objects, and a running wheel) on heroin-induced addiction-like behaviors and on the vulnerability to relapse following abstinence. We hypothesized that rats housed in an enriched environment will exhibit less addiction-like behaviors for heroin and will be more resilient to drug-induced relapse than standard-housed rats.

**Molecular Effects of Drugs and Environment**

Drugs of abuse have profound structural and functional effects on neurobiology (Koob and Volkow, 2016) that can endure long after drug-use has ceased and may be driven by molecular and epigenetic mechanisms (Nestler, 2013). Previously, we reported mRNA changes that were associated with incubation of heroin seeking during enforced abstinence (Kuntz-Melcavage et al., 2009; Kuntz et al., 2008a). Here, we extend these findings by exploring the hypothesis that epigenetic regulation of differentially-expressed genes is associated with enrichment and drug-self administration and abstinence.

Although every cell within an organism contains the same genetic code, epigenetic mechanisms (DNA and chromatin modifications, and transcription factor expression/activation) orchestrate genomic structure and availability, creating cell- and state-specific transcriptomes. In the central nervous system (CNS), epigenetic mechanisms are not only essential to development and cell differentiation (Keverne et al., 2015), but also responsivity to the environment throughout
adolescence and adulthood (Cholewa-Waclaw et al., 2016). DNA modifications and specifically cytosine methylation (mC) have received special attention as an epigenetic mechanism in drug addiction (Nielsen et al., 2012). mC is the most common covalent DNA modification. While traditionally viewed solely as a repressive mark that occurred in only CG dinucleotide motifs, the field has evolved to the understanding that non-CG methylation (termed CH, where H is an A, C, or T) is common and that methylation can be associated with both decreased and increased gene expression (He and Ecker, 2015; Kinde et al., 2015). In human heroin abusers, altered methylation of the µ opioid receptor has been found in blood samples (Nielsen et al., 2010; Nielsen et al., 2009) and across a number of genomic regions in prefrontal cortex (Kozlenkov et al., 2017). In vitro morphine treatment causes genome-wide hypomethylation (Trivedi et al., 2014). However, to our knowledge, no studies have examined DNA methylation in controlled animal self-administration models of heroin addiction. Therefore, in this study, analyses of genome-wide, repeat region, and specific gene promoter methylation levels were performed, taking advantage of recent technical advances that greatly improve the rigor of epigenetic studies (Masser et al., 2017).
Materials and Methods

Behavioral Experiments

Subjects and Housing Conditions

The animal treatment paradigm is summarized in Figure 6-1A. Prior to the start of the experiment, 31 adult male Sprague-Dawley (Charles River) rats were randomly assigned to 2 housing conditions for the entire duration of the study. In the Standard condition, rats (n=16) were singly housed in typical suspended, stainless steel cages. In the Enriched Condition, rats (n=15) were group housed (2-3 subjects/enlarged cage) with novel objects presented daily and continuous access to a 12 inch exercise wheel. Food and water were available ad libitum unless stated otherwise. All studies were approved by the Penn State College of Medicine, Institutional Animal Care and Use Committee.

Surgeries

Following 2 weeks in either housing condition, each rat was implanted with an intravenous jugular catheter as described previously (Grigson et al, 2002). The rats were given 1 week to recover prior to the start of testing in their previously assigned housing environment. General maintenance of catheter patency included daily examination and flushing of catheters using heparinized saline (0.2 ml of 30 IU/ml heparin). Catheter patency was verified when necessary using 0.2 ml of propofol (Diprivan 1%) administered IV.
Procedure

Apparatus: Testing was conducted in 12 self-administration chambers as previously described (Puhl et al, 2013). A stimulus light was located 6 cm above each tube. Using a lickometer circuit, rats were placed on a fixed ratio 10 (FR10) schedule of reinforcement where completion of the 10 lick requirements on the inactive spout elicited no consequence, and completion of the 10 lick requirement on the active spout led to an IV infusion.

Acquisition: Rats were habituated to the self-administration chambers 5 min per day for 2 days prior to the start of acquisition. During habituation, the rats were on a water restriction regimen in which they received 5 min access to water through one of the two spouts in the chambers and 25 ml of water in the home cage overnight. This habituation occurred over 2 days until each animal experienced each of the 2 spouts. Thereafter, water was returned to the rats and acquisition of drug self-administration began. During acquisition, the 2 empty spouts advanced and the drug session started. Rats were placed on a fixed ratio 10 (FR 10) schedule of reinforcement where 10 licks on the rightmost empty active spout resulted in a 6 sec IV infusion of either saline (n=11) or 0.06 mg/0.2ml of heroin (n=20) as previously described (Kasprowicz et al, 2008). Drug or saline delivery was signaled by offset of the stimulus light and onset of the tone and house light, which remained on for a total of 20 sec, as well as the retraction of both spouts. Further responding during this time was not reinforced. The access period for heroin was 3 h/day and there was a total of 12 such days of acquisition.

Progressive Ratio Challenge: Upon completion of trial 12 of the drug acquisition phase, a single PR test (progressive ratio test) was conducted on the following day to assess motivation as previously described in (Imperio et al, 2015). On the PR test, each subject began on an FR 10 on the active spout for the 1st infusion, with subsequent infusions requiring the completion of an increasing number of operant responses. Specifically, animals were required to successively
execute the following response schedule to receive sequential infusions: 10, 12, 16, 22, 30, 40, 52, 66, 82, 100, 120, 142, 166, etc. Breakpoint was defined as the last ratio completed. The trial terminated when 30 min elapsed without having earned an infusion.

**Maintenance Trial:** After the single PR test, all rats were given one additional drug access trial on the FR 10 schedule of reinforcement to reestablish drug-taking behavior prior to the extinction and reinstatement test.

**Drug Abstinence:** Following the single maintenance trial, all rats underwent 14 d of enforced home cage abstinence. During this time, all rats were left undisturbed in their home cages, except for typical maintenance such as weighing, flushing of the catheters, and changing of the novel objects for the enriched conditions.

**Extinction & Reinstatement Test:** *Extinction.* Upon completion of the abstinence phase, all rats participated in a one-day extinction and reinstatement test as detailed previously (Imperio et al, 2015). During the extinction phase, all rats underwent a 6 h extinction session where responding on the active empty spout was not rewarded with an infusion, but all cues previously paired with the drug were presented each time 10 responses were made. However, responding (licks/contacts) was monitored for behavioral analysis. *Reinstatement.* Immediately following the 6 h extinction test, rats received a single, computer-delivered non-contingent IV infusion of 0.06 mg/0.2ml of heroin and responding on the active and inactive spouts was recorded for another h with no drug availability.

**Drugs**

Heroin HCl was generously provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, N.C., USA). Heroin was dissolved in sterile physiological saline to a concentration of 0.3 mg/ml.
**Behavioral Data Analysis**

The behavioral data were analyzed using a mixed factorial analysis of variance (ANOVA). The between-group factors for all analyses was housing condition (standard or enriched) and drug (saline or heroin). Drug intake over the acquisition phase was measured using a 2x2x12 (housing x drug x trials) mixed factorial ANOVA varying standard saline (SS), enriched saline (EH), standard heroin (SH), enriched heroin (EH). Drug-seeking behavior under extinction conditions across the 6 hours was similarly measured using a 2x2x6 mixed factorial ANOVA. Data from the progressive ratio test and reinstatement behaviors were analyzed using a 2x2 ANOVA at the relevant time points. Planned comparisons using standard t-test were conducted between the standard heroin vs. enriched heroin groups for all behavioral tests. Post hoc tests were conducted, when significant interactions were observed, using Fisher’s least significant difference (LSD) tests, with \( \alpha \) set at 0.05.

**Sacrifice and Tissue Collection**

Rats were decapitated 24 hours after reinstatement testing to ensure that all drug was metabolized. Brains were rapidly removed from skulls and the nucleus accumbens containing core and shell (Nac), ventral tegmental area (VTA), and medial prefrontal cortex (mPFC) were dissected manually as previously described (Kuntz et al, 2008a) (Freeman et al, 2001a). Both hemispheres of each brain region was then placed in pre-chilled tubes, and immediately frozen in liquid nitrogen and stored at -80°C.
Molecular Studies

RNA & DNA Isolation

DNA and RNRNA were co-isolated from brain tissues using the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) (Mangold et al, 2017a). Quantity and quality of DNA and RNA was assessed spectrophotometrically and by capillary electrophoresis (Agilent Bioanalyzer).

qRT-PCR for Gene Expression

Quantitative PCR (qRT-PCR) analyses of genes of interest were performed using TaqMan Assay-On-Demand (Life Technologies, Carlsbad, CA) gene-specific primers/probe assays (See Table 6-1) and a QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems) as previously described (Mangold et al, 2017b). Genes of interest were selected based on previous results demonstrating heroin responsiveness (Kuntz-Melcavage et al, 2009; Kuntz et al, 2008a). Relative gene expression was calculated with Expressionsuite v 1.0.3 software using the 2^−ΔΔCt analysis method with β-actin as the endogenous control. Gene expression data were analyzed using a 2-way ANOVA varying environment and drug. Posthoc tests were performed, where appropriate, using Tukey’s pairwise comparison. Planned comparisons were performed for all groups using a standard t-test and Bonferroni correction.

Genomic Repeat Element Analysis

Methylation levels of repetitive elements were examined as a surrogate for genome-wide methylation changes. Genomic DNA (n=5-10/group) was bisulfite-converted using the EZ DNA methylation-lightening kit (Zymo Research, Irvine, CA) and then subjected to PCR amplification
using bisulfite-specific converted primers for LINE-1 and ID elements (EpigenDx, Hopkinton, MA). Following PCR amplification, amplicons were analyzed for CG methylation via pyrosequencing (EpigenDx, Hopkinton, MA) (Hadad et al, 2016).

**Whole-Genome Bisulfite Sequencing**

Whole-genome bisulfite sequencing was performed using Swift accel-NGS methyl-seq library kit (swift bioscience) per manufacturer instruction and as previously described (Hadad et al, 2016). gDNA was sheared to ~350bp fragments using sonication (Covaris, E220) and assessed for fragment size by DNA capillary chip electrophoresis (Agilent, DNA 1000). 100ng Fragmented DNA was bisulfite converted using EZ DNA methylation-Gold kit (Zymo Research) and denatured at 95°C for 2 minutes and placed on ice for 2 additional minutes. Bisulfite converted DNA was than adapter ligated and indexed by PCR per manufacturer recommendations. Libraries were quantified by PCR (KAPA library quantification kit, KAPA Biosystems), diluted to 2nM and pooled at equimolar concentration prior to sequencing. Pooled samples were loaded at 12 pM and sequenced using 100 bp pair-end reads on illumina HiSeq2500 platform.

Sequence reads were adapter trimmed and an additional 3 bp were trimmed off the 3’ and 5’ ends using CLC Genomics Workbench v10.1.1 (RRID:SCR_011853). Trimmed reads were than filtered for Q-score (> 30) and ambiguous nucleotides (< 2). Computational analysis was performed in UNIX and R using custom scripts. Reads were mapped to the *Rattus norvegicus* reference genome (rn6, RGSC Rnor_6.0, UCSC Genome Browser) using bismark bisulfite mapper (Krueger and Andrews, 2011). Methylation calls for mapped reads were extracted using bismark methylation extractor and used for methylation averages and description of methylation differences.
Genome-wide averages in methylation were calculated as the average methylation call over all cytosines (CGs and CHs). For methylation levels of transposable elements, the most abundant transposable element (TE) families (LINEs, SINEs, LTRs and DNA transposons) in the *Rattus norvegicus* genome were quantified using RepeatMasker (Smit and Hubley 2014). A complete rodent library was used to accurately identify species specific TEs. The settings -s and –nolow were utilized to increase sensitivity and exclude any low complexity regions from the search. All homologous regions were kept and considered as transposable element hits. Bed files for each TE family containing chromosomal coordinates were then created from the RepeatMasker output file and overlapped with methylation calls coordinates using bedtools (Quinlan and Hall, 2010). Sites overlapping with annotated repeats were than averaged to determine overall methylation for each transposable element family.

Differences in methylation patterns of CG Islands, shore and shelf and promoters between treatment groups were assessed using a tiling approach. Average of non-overlapping 100 bp windows for promoters and annotated CGI units was computed and compared between groups for each of the brain regions. Promoters were defined as the TSS ± 2 kb flanking region of all annotated rat protein-coding genes included in the RefSeq database. Shores and shelf were defined as ±4 kb from the CGI and were calculated based on the position relative to annotated CGIs.

**Targeted DNA Methylation Analysis Using Bisulfite Amplicon Sequencing (BSAS)**

To analyze DNA methylation of EGR1 and EGR2 (as well as OPRM1) with base resolution for both CpG and CH contexts, BSAS was performed as previously described (Masser *et al.*, 2013; Masser *et al.*, 2015). Briefly, isolated genomic DNA from the mPFC and NAc was bisulfite-converted using the EZ DNA methylation-lighting kit (Zymo Research, Irvine, CA). Following bisulfite conversion, selected regions of the genes of interest were amplified with primers (Table
2) designed against the converted sequences using MethylPrimer Express Software (Life Technologies, Carlsbad, CA) with the stipulation that the primer binding region contained no CpG sites. Amplicons were isolated by column purification (Qiagen) and then equimolar amounts of the amplicons from each subject were pooled. Sequencing libraries for each sample were then prepared by tagmentation (Nextera XT, Illumina), with unique dual indexes for each subject. Libraries were then pooled and sequenced, at paired-end 150bp, using an Illumina MiSeq. Data were saved as FASTQ files and imported into CLC Genomic Workbench version 7.5.02 for alignment and subsequent analysis. Reads were trimmed (Q<30) and aligned to in silico converted reference sequences for EGR1, EGR2 and OPRM1. Low frequency variant detection was performed to quantify methylation levels at each CpG site in the amplified regions. Only CpG sites with >50X coverage were considered to have sufficient depth for accurate quantitation. CpG methylation was then analyzed in both base-specific and regional manners.
Table 6-1. Genes chosen for qRT-PCR analysis.

Gene selection for differential mRNA analysis was based on previously published studies of mRNA differences associated with heroin SA and seeking (Kuntz-Melcavage et al, 2009; Kuntz et al, 2008a; McFalls et al, 2016).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Assay Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drd2</td>
<td>dopamine receptor D2</td>
<td>Rn00561126_m1</td>
</tr>
<tr>
<td>Crh</td>
<td>corticotropin releasing hormone</td>
<td>Rn01462137_m1</td>
</tr>
<tr>
<td>Pdyn</td>
<td>prodynorphin</td>
<td>Rn00571351_m1</td>
</tr>
<tr>
<td>EGR1</td>
<td>early growth response 1</td>
<td>Rn00561138_m1</td>
</tr>
<tr>
<td>EGR2</td>
<td>early growth response 2</td>
<td>Rn00586224_m1</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
<td>Rn00561681_m1</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
<td>Rn00560868_m1</td>
</tr>
<tr>
<td>OPRM1</td>
<td>opioid receptor, mu 1</td>
<td>Rn01430371_m1</td>
</tr>
</tbody>
</table>

Table 6-2. Primers designed to target specific regions of EGR1, EGR2 and OPRM1 in the BSAS experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Region of Interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR1</td>
<td>5’AATTAGTAGTTAAAAAGTTTTTGGTT 3’</td>
<td>5’TCCCTCCTACTCCTTATATAAT 3’</td>
<td>Promoter Region (EGR1-1)</td>
</tr>
<tr>
<td>EGR1</td>
<td>5’ATTATAAGGAGTAGGAAAGAGTTT 3’</td>
<td>5’ACCTCTATTTCAAAAAATCTAAAAC 3’</td>
<td>Promoter Region (EGR1-2)</td>
</tr>
<tr>
<td>EGR1</td>
<td>5’ATTTAAAAATGGAGAGAGATGAT 3’</td>
<td>5’TCTACACAATACTCCATAACAT 3’</td>
<td>TSS/Exon1 (EGR1-3)</td>
</tr>
<tr>
<td>EGR1</td>
<td>5’AGTGGTAATATTTTGTTGTTGA 3’</td>
<td>5’TAAAACCTAATAACACCCCTT 3’</td>
<td>Exon 2 (EGR1-4)</td>
</tr>
<tr>
<td>EGR1</td>
<td>5’AGGGGTGGTTTATTAGGT1TTT 3’</td>
<td>5’CAAACCTCAAACCAACACAT 3’</td>
<td>Exon 2 (EGR1-5)</td>
</tr>
<tr>
<td>EGR2</td>
<td>5’AGGATGGGTTTTAAAGGAGATGGT 3’</td>
<td>5’CAAATCTATTCTATATACTCCCA 3’</td>
<td>Exon 2 (EGR2-1)</td>
</tr>
<tr>
<td>EGR2</td>
<td>5’TGTGGATGGAGTGATTTAAGAAGA 3’</td>
<td>5’TAACCATCTTCCCAATAATAA 3’</td>
<td>Exon 2 (EGR2-2)</td>
</tr>
<tr>
<td>EGR2</td>
<td>5’TATATGGATTGAGGAAATAGGGT 3’</td>
<td>5’CTCCAAACAAACAACTAAAA 3’</td>
<td>Promoter Region (EGR2-3)</td>
</tr>
<tr>
<td>OPRM1</td>
<td>5’TTTTGGTGTATTAGGGTTG 3’</td>
<td>5’ACCAAAACCAAATACTAAA 3’</td>
<td>Promoter Region</td>
</tr>
</tbody>
</table>
Results

**Drug Intake**

Total drug taking across the entire acquisition period was compared between standard environment and enriched environment groups. No differences were observed in the total drug-taking between groups over the two weeks of self-administration (Figure 1B).

**Motivation**

To examine the subjects’ addiction-like behavior, their motivation to work for drug was assessed by a progressive ratio test (Figure 1C). When challenged with a single progressive ratio test, significant main effects of housing (p<0.01) and drug were observed (p<0.01). Planned comparisons between SH and EH revealed that rats housed in the enriched condition were less willing to work for heroin than were rats housed in the standard condition (p<0.05).

**Extinction and Reinstatement**

To reestablish FR responding, a single drug maintenance day was conducted prior to 14 d of enforced abstinence. After 14 days of enforced abstinence, a significant overall difference was seen among the 4 groups in drug-seeking behavior when no drug was available during the 6 h extinction test (Figure 6-1D) (2 x 2 x 6 ANOVA, Housing p=0.10; Drug, p<0.001; Trials, p<0.001; Housing x Drug x Trials, p=0.56). Planned comparisons revealed that enriched rats showed a significant decrease in drug-seeking behaviors compared to standard heroin rats within
Figure 6-1. – Addiction-like behavior.

Rats were split between a standard and enriched environment for the duration of the study (A). After two weeks, catheter surgery was performed followed by habituation to the test chambers and recovery for two days. Self-administration lasted for two weeks, at the end of which the progressive ratio test was performed. Following two weeks of abstinence, extinction reinstatement testing was performed for a day. Euthanasia was the next day, to ensure no drug was remaining at the time of tissue collection. (B) Number of heroin (0.06 mg/0.2ml of heroin) infusions/3 h for standard (SE) heroin and enriched (EE) heroin across 12 trials. (C) Number of saline or heroin infusions earned during progressive ratio testing for standard saline, enriched saline, standard heroin, and enriched heroin. (D) Number of drug seeking behaviors/1 h exhibited during the 6 h extinction and (E) 1 h heroin induced reinstatement tests for standard saline, enriched saline, standard heroin, and enriched heroin. *p<0.05, ANOVA n=5-10/group, data are presented as mean (+/- SEM)
the 1st hour of extinction (p<0.05). No differences in extinction responding were observed from hour 2 to hour 6.

When primed with a single non-contingent infusion of heroin (Figure 6-1E), there was a significant difference in resumption of drug-seeking behaviors among the conditions. The main effect of Drug was significant, (p<0.001), showing greater drug-induced reinstatement behavior by rats with a history of heroin vs. saline self-administration. Housing condition also had a significant main effect (p<0.05) although the Housing x Drug interaction did not (p=0.16). No significant interaction effect was found between environment and drug. Planned unpaired t-tests were conducted to determine the differences in reinstatement responding between environmental enrichment paradigms. The enriched heroin rats demonstrated less reinstatement responding as compared to standard heroin rats (t(18)=2.28, p<0.05).

**Global DNA methylation**

**Total Genomic Methylation**

Both cocaine (Novikova *et al*, 2008; Tian *et al*, 2012) and morphine (Trivedi *et al*, 2014) have been reported to decrease total methylation levels across the genome. To assess whether heroin, in the context of *in vivo* drug self-administration, decreases total DNA methylation, we performed low coverage whole genome bisulfite sequencing (Hadad *et al*, 2016). This approach harnesses the quantitative accuracy of bisulfite sequencing by generating millions of methylation counts (a sequencing read over a cytosine) per sample, and discriminating between CG and CH contexts (Masser *et al*, 2017).
Low coverage whole genome bisulfite sequencing was performed for VTA, mPFC, and NAc for each of the four groups (n=3/group). An average of $26.7 \times 10^6 \pm 5.5 \times 10^6$ CG and $5.5 \times 10^9 \pm 1.2 \times 10^9$ CH counts were collected across the genome. Average levels of CG methylation were 74.3-75.8% across all treatment groups and brain regions. No differences in CG methylation across the whole genome (Figure 6-2A) were evident, nor were differences observed when counts were restricted to promoters (defined as $\pm 2$kb the transcription start site of every annotated gene in the rat genome) (Figure 6-2B) or across all 18,411 annotated CG island/shores/shelves (Figure 6-2C). When plotted as profiles across the genome regions, very consistent patterns – lowest at transcription start sites and CG islands with higher methylation in flanking regions – were observed across treatments and between brain regions.

It is possible that the differences in whole genome methylation previously reported represent CH methylation as this is high in the CNS and typically ELISA and HPLC based methods for mC quantitation do not differentiate between CG and CH methylation. CH methylation averages were lower than CG across the genome, as would be expected (Figure 6-3A). No differences with treatment in any brain region were evident at whole genome level or when restricted to genic regions (defined as gene coordinates for all refseq annotated genes) (Figure 6-3B).

**Repeat Element Methylation**

Previous studies have shown that LINE and ID repeat elements specifically could be altered with opiate exposure (Trivedi *et al*, 2014), thus, we extracted methylation counts from repeat elements in the rat genome. Furthermore, given that repeat elements constitute a large portion of the genome (Trivedi *et al*, 2014), they have been proposed as a marker for global DNA methylation.
Figure 6-2. – CG methylation levels across whole genome, and promoter and CG island regions.

Low coverage whole genome bisulfite sequencing was performed across VTA, NAc, and mPFC brain regions. (A) Total methylation counts / total counts for CG was used to determine the average genomic methylation per sample (n=3/group). (B) Profiles of the average CG methylation across all annotated promoters were generate for +/- 2kb of the transcription start site (TSS). (D) Similarly, average CG methylation profiles for all annotated CG islands, shores and shelves were calculated per animal. No differences across the whole genome, promoters or CG island unites were observed.
Repeat element methylation was extracted from the low coverage whole genome bisulfite sequencing data for LINEs, SINES, and LTRs in both C (A-C) and CH (D-F) contexts. No significant differences were observed n=3/group.
Figure 6-4. – LINE-1 and ID element methylation.

LINE-1 and ID element methylation was also assessed by pyrosequencing. No differences in methylation were found. ANOVA n=5-10/group.
Low coverage whole genome bisulfite sequencing was performed across VTA, NAc, and mPFC brain regions and methylation at non-CG, CH, sites was determined. (A) Total methylation counts / total counts for CH was used to determine the average genomic methylation per sample (n=3/group). (B) Average CH methylation across all genic regions (inclusive or annotated promoters, introns, and exons) were computed for each sample. No differences across the whole genome, promoters or CG island unites were observed.

Figure 6-5. – CH methylation levels across whole genome and specifically to genic regions.
(Doehring et al, 2013; Yang et al, 2004). LINE, SINE (short interspersed elements), and LTR (non-long terminal repeat retroposons) were extracted from the whole genome bisulfite sequencing data. mCG levels were high (>80%) and extremely consistent between treatments and across brain regions (Figure 6-5A-C). mCH levels in repeat elements were lower (~2%) but did not differ between brain regions or treatments (Figure 6-5D-F). Previous reports used pyrosequencing to examine repeat elements (Trivedi et al, 2014) and therefore repeat elements (LINE-1 and ID) were also examined by pyrosequencing. Again, no differences were observed between the four treatment groups, either as individual positions or as averages (Figure 6-6). As previous experiments have also used ELISA based approaches, these were also performed for mPFC and NAc with no differences evident (data not shown). Together the whole genome bisulfite sequencing, pyrosequencing, and ELISA data demonstrate that there are no changes in total methylation with environmental enrichment or heroin self-administration across the mesolimbic pathway. Data were in fact quite consistent and nothing approaching the >50% decrease reported in vitro was observed (Trivedi et al, 2014).

**Gene Expression**

The analysis of total genomic and repeat element methylation indicates that these are not altered with environmental enrichment or heroin self-administration. Gene expression responses to opiates have been reported across the mesolimbic pathway (Cooper et al, 2017; Sanchez et al, 2016). To determine the relationship between drug exposure and enrichment status, gene expression analysis was conducted on the VTA, mPFC, and NAc using 8 targets of interest that have been shown to be altered due to heroin or abstinence (Kuntz-Melcavage et al, 2009; Kuntz et al, 2008a).
EGR1 gene is an early response gene that has been shown to be by our laboratories (Kuntz et al., 2008a) and others (El Rawas et al., 2009) to be sensitive to substance abuse. In the present study, EGR1 expression in the mPFC (Figure 6-6A) demonstrated a significant interaction of heroin and environment (p<0.05). Post-hoc pairwise analysis revealed that enriched heroin subjects displayed significantly lower amounts of EGR1 expression in the mPFC as compared to the other three groups. In the NAc, EGR1 expression demonstrated an interaction effect (p<0.05) of housing and drug. Post-hoc testing revealed higher EGR1 expression in the standard saline group as compared to all other groups. No differences in EGR1 in the VTA were observed.

EGR2 is also an early response gene that increases in expression following heroin abstinence (Kuntz et al., 2008a) and has been reported to be responsive to other drugs of abuse (Cadet et al., 2013). In the mPFC EGR2 expression demonstrated significant main effects of housing (p<0.001) and drug (p <0.05) and no interaction effect (Figure 6-6B). Planned post-hoc pairwise comparisons showed that expression was lower in the enriched heroin group as compared to all other groups. In the NAc, significant main effects of housing (p <0.01) and drug (p <0.05) and no interaction effect was again observed, with higher EGR2 expression in the standard saline group as compared to all other groups. EGR2 expression did not vary between treatment groups in the VTA. OPRM1 was also examined across all of the treatment groups and brain regions, but no differences were found between groups (Figure 6-6C). Expression of Drd2, Crh, Pdyn, Npy, and Bdnf were not significantly different among the four conditions in any of the brain regions examined (data not shown).

To further understand the relationship between gene expression and behavior due to heroin exposure, behavioral measures were correlated to gene expression in the heroin groups. For EGR1, a significant relationship was seen between mPFC qPCR and reinstatement behaviors (r=0.54;
Relative gene expression was assessed via QRT-PCR on genes EGR1, EGR2 and OPRM1 in the VTA, mPFC and NAc. (A) Differential expression of EGR1 was observed between SS and EH, ES and EH, and SH and EH in the mPFC. Higher expression of EGR1 was observed in the SS group relative to ES, SH and EH. (B) Differential EGR2 expression was observed between SS and SH, ES and EH, and SH and EH in the mPFC. In the NAc, SS had higher expression of EGR2 relative to EH and SH. (C) No differences in OPRM1 expression were observed in the VTA, mPFC or NAc \( *p<0.05 \), \( **p<0.01 \), \( ***p<0.001 \), (B) ANOVA \( n=5-10/group \) (standard saline = SS, enriched saline = ES, standard heroin = SH enriched heroin = EH) behavioral measurements.
p<0.05) and NAc EGR1 expression with first hour extinction seeking behavior (r=0.51; p<0.05).
EGR2 in all regions of interest showed no significant correlations with any of the behavioral measures.

Site-Specific DNA Methylation via BSAS

EGR1 Cytosine Methylation

The lack of a significant difference in global DNA methylation status is not surprising given that such changes would be indicative of a change in cell status (e.g., cancer, stemness). However, given that expression of EGR1 and EGR2 genes was observed to change with housing environment and heroin self-administration in the mPFC and NAc, DNA methylation at these specific genes was examined in a base-specific manner in promoter and intragenic regions. Regions of interest for EGR1 (Figure 6-7A) were selected based on the data shown above and published work showing that EGR1 methylation status is altered with reward learning (Day et al, 2013). mCG levels were very low (<5%) in promoter regions and the first exon (Figure 6-7B&C). Within the second exon, mCG levels were higher and showed a consistent pattern between the NAc and mPFC at specific CG sites. Mean CG methylation in the analyzed regions did not differ between treatment groups. Utilizing the base-by-base resolution of BSAS, we employed a 2-way ANOVA (housing x drug), with the additional criteria that methylation differences exceed 5% (in order to uncover differences of physiological relevance, not just statistical significance). In exon 2 (EGR1-4, EGR1-5) of the NAc, two-way ANOVA revealed a significant main effect of Drug. Planned comparisons were conducted at sites 1429, 1445, 1476, 1487, and 1555 were found to have significant differences in DNA methylation among the four groups. (p<0.05) (Figure 6-7B and Table 6-3). In the second
exon region of mPFC tissue, no sites were found to be differentially methylated among the four groups (Figure 6-7C and Table 6-3).

As BSAS quantifies methylation at both CG and CH, methylation at CH sites of EGR1 promoter and intragenic regions were also examined. mCH was extremely low across the promoter regions and first exon with higher levels in second exon region. Reproducible, site-specific CH methylation patterns were observed in both NAc and mPFC (Figure 6-7D&E). No mean differences in mCH methylation were observed. Analysis of CH methylation using 2-way ANOVA revealed increased methylation at site 1461 in the standard heroin group in the NAc (Figure 6-7D and Table 6-3). No differences in mCH were observed in the mPFC. EGR2

**EGR2 Cytosine Methylation**

For EGR2, regions from the promoter, intronic, and exonic regions were analyzed by BSAS (Figure 6-8A). CG methylation was low in the promoter region and across the first exon with higher methylation in the second exon in both the NAc and mPFC (Figure 6-8B&C). Methylation of CG sites was higher in standard saline than other groups across the EGR2 promoter in the mPFC (Figure 6-8C), (EGR2-1) (F\(_{3,21}\)=4.12; \(p<0.05\)). Post hoc tests indicated that the standard saline promoter methylation was hypermethylated compared to the other 3 conditions (\(p<0.05\)). Higher methylation in the standard saline group was further supported at specific CG sites in the EGR2 gene promoter region in the mPFC using 2-way ANOVA as employed in EGR1 (environment x drug, 5% methylation difference cut-off). Greater methylation was observed in the standard saline group relative to the standard heroin and enriched heroin groups (\(p<0.05\)) at sites -394, -346, -265, -221, -202, -180, -175, and -118 (Figure 6-8C and Table 6-3). Site 2477 (exon 2) of EGR2 in the
mPFC was also found to be differentially methylated between groups (Figure 6-8C). No differences were observed in the NAc.
Figure 6-7. CpG and CH methylation of EGR1 in the mPFC and NAc.

Bisulfite amplicon sequencing (BSAS) was used to assess site-specific methylation in EGR2. (A) Representation of EGR2 gene (promoter and gene body) and BSAS primer placement (1-1, 1-2, 1-3, 1-4, 1-5). Site-specific differences in methylation were observed in within the gene body of EGR1 in the NAc. (C) No site-specific methylation differences were observed in the EGR2 in the mPFC. (D) Higher CH methylation at site 1461 was observed in the NAc of the enriched saline group. (E) No differences in CH methylation were observed in the mPFC. 2-way ANOVA, n=5-10/group.
CH was low across EGR2 with specific sites demonstrating higher levels of CH methylation (Figure 6-8D&E), but with some inter-animal variability. Differential methylation analysis by 2-way ANOVA revealed only one site - 569 - within the NAc (Table 6-3). No differences in mCH were found in the mPFC.

Even through different expression of OPRM1 was not observed, the methylation status of OPRM1 was also analyzed given previous human literature showing a difference in DNA-methylation in former opiate addicts (Nielsen et al, 2009). Analysis of the OPRM1 gene promoter region (data not shown) revealed no significant differences among the 4 conditions ($p>0.05$). Closer inspection at each individual CG site further confirmed this lack of difference in DNA methylation. Regardless of enrichment status or heroin exposure, all subjects showed similar methylation status at each CG site along the promoter region ($p>0.05$).

Given that DNA methylation has been shown to affect mRNA expression, correlational analyses were also conducted to determine if changes in DNA methylation at various CG sites in the EGR1, EGR2, and OPRM1 were associated with the mRNA changes seen above. No significant difference was found at any of the CG sites among the 3 genes relating to changes in mRNA expression. Regardless of condition or drug exposure, no significant correlation was found between mRNA expression of any of the 3 genes and methylation status of a specific CG site. However, it is worth noting that in the NAc, the lowest levels of methylation (standard saline) of EGR1 (Figure 6-7) were matched with the highest mRNA expression (Figure 6-6).
Figure 6-8. – CpG and CH methylation of EGR2 in the mPFC and NAc.

Bisulfite amplicon sequencing (BSAS) was used to assess site-specific methylation in EGR2. (A) Representation of EGR2 gene (promoter and gene body) and BSAS primer placement (2-1, 2-2, 2-3). No site-specific differences in methylation were observed in EGR2 within the NAc. (C) Site-specific methylation differences were observed in the promoter region and gene body of EGR2 in the mPFC. (D) Higher CH methylation at site 569 was observed in the NAc of the standard saline group. (E) No differences in CH methylation were observed in the mPFC. 2-way ANOVA, n=5-10/group.
Table 6-3. Sites in EGR1 and EGR2 found to be significantly different in mPFC and NAc.

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<tr>
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<th>Enriched Saline mean</th>
<th>Standard Heroin mean</th>
<th>Enriched Heroin mean</th>
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**Discussion**

With the current epidemic of opiate addiction, an understanding of the environmental factors that can prevent or aid treatment of opiate addiction is needed. Additionally, insight into the molecular mechanisms of opiate addiction may provide novel targets for pharmacological intervention to prevent addiction or aid abstinence. In these studies, we found that environmental enrichment, while not reducing heroin self-administration in acquisition, significantly reduced motivational measures of drug seeking and drug taking. At the molecular level, expression in specific immediate early genes, EGR1 (also known as Zif268 or NGFI-A) and EGR2 (also known as Krox20), is evident with heroin self-administration and the expression of EGR1 was found to correlate with both heroin-seeking (mPFC) and heroin-induced reinstatement (NAc) behavior. Epigenetically, heroin self-administration and abstinence is not accompanied by widespread changes in genomic methylation. Site-specific responses in methylation within the EGR1 and EGR2 promoter and intragenic regions, however, are responsive to environment and drug variables.

**The Effect of Enrichment on Addiction-Like Behaviors**

The interaction between genes and the environment has a tremendous impact on the behavioral response to drugs of abuse. Positive and negative experiences in a person’s life can alter the susceptibility to take and develop dependence and addiction to drugs of abuse. Contrary to previous studies using cocaine from our laboratory (Puhl *et al.*, 2012), the present data demonstrate that environmental enrichment in adult rats does not have an effect on acquisition of heroin self-administration. When challenged with a progressive ratio test, however, enriched rats showed a significant decrease in their willingness to work for drug compared to standard housed heroin rats. Furthermore, we show that enriched rats also exhibited less drug-seeking behaviors in the first hour
of extinction testing following a prolonged period of enforced abstinence compared to standard housed rats and a decreased reinstatement of drug seeking after a non-contingent drug prime. Environmental enrichment, then, reduced addiction-like behavior for heroin in heroin-experienced adult rats.

Beneficial effects of environmental enrichment such as a reduction in stress and reduced anxiety have been proposed as protective mechanisms by which environmental enrichment might counteract the deleterious effects of drugs of abuse (Solinas et al., 2010). Indeed, the availability of an alternative reward such as a sweet or exercise has shown great promise in combating the various stages of addiction at the clinical and preclinical levels (Brown et al., 2009; Lenoir et al., 2013b; Liu and Grigson, 2005; Lynch et al., 2010). Yet, in the present study, both enriched and standard housed subjects infused the same amount of drug. A number of factors could contribute to environmental enrichment not reducing drug taking. First, the Puhl et al. study intentionally employed a very low dose of cocaine, while this study employed a standard to high dose of heroin. Environmental enrichment may have reduced acquisition of heroin self-administration if a lower dose of drug had been used. Another factor may relate to the use of a short-access (3 h) procedure that is known to produce fairly stable drug-taking behavior (Ahmed and Koob, 1998). With a longer access paradigm differences in intake may become evident. Centrally, these prior studies examined cocaine self-administration and, relative to cocaine, heroin may have a higher abuse liability (Madsen and Ahmed, 2015).

A central finding was that environmental enrichment reduced cue-induced seeking and extinction behavior. This is similar to findings with sucrose self-administration; an effect that was attributed to enhanced learning ability (i.e., regarding the discontinued availability of a reward) (Grimm et al., 2008). Indeed, others have found that environmental enrichment can have a powerful effect on learning and memory, as well as increasing neurite branching and synapse formation.
(Diamond et al, 1976; Diamond et al, 1966; Walsh et al, 1969). It is possible that our study reflects the beneficial effect of environmental enrichment on neuronal and behavioral plasticity.

The relationship between environmental enrichment and addiction may be bi-directional. Although loss of interest in rewards and personal responsibilities is a hallmark symptom of addiction, natural rewards can serve as a means to treat and protect the individual (Grigson, 2008). The availability of alternative rewards has profound effects on drug-taking behavior, as demonstrated by a brief exposure to a sweet solution during drug abstinence can reduce resumption of cocaine-seeking behaviors (Liu et al, 2005). Additionally, concurrent choices of an alternative reward along with drug access can protect against drug taking (Carroll et al, 2008; Lenoir et al, 2013a). The importance of alternative rewards can further be shown during the loss of enrichment status before drug exposure. Mice that had previously been housed in an enriched environment prior to drug exposure had increased sensitivity to the rewarding properties of cocaine when the enriching environment was removed (Nader et al, 2012).

These findings are bolstered by clinical studies using alternative rewards to combat addiction. Inclusion of aerobic exercise during treatment can help promote drug abstinence in recovering alcoholics (Brown et al, 2009). Availability of monetary rewards can attenuate the choice of heroin self-administration (Comer et al, 1997). Therefore, in addition to the neurobiological benefits of environmental enrichment, the existence of an alternative reward can reduce drug-taking behaviors and aid in the treatment of drug addiction. This was seen here when the rats were challenged on a PR test, during extinction testing, and during drug-induced reinstatement. Exposure to an enriched environment significantly reduced the willingness to work for heroin, significantly reduced seeking during the first hour of extinction, and significantly reduced heroin-induced reinstatement of heroin-seeking behavior relative to standard housed controls with an identical history of heroin self-administration, even though both heroin taking groups had equivalent drug taking histories.
Global Methylation

One of the most intractable aspects of drug addiction is relapse to drug taking, even after prolonged periods of drug abstinence. Stressors and environmental factors can contribute to relapse, but a neurobiological basis of relapse has proven difficult to identify and modulate. Epigenetic factors have emerged as a potential mechanism for addiction’s persistence due to their perceived stability and thus there is a growing interest in the interaction of epigenetics and drug-abuse (Nestler, 2014). In studies of non-contingent cocaine administration, decreased methylation in the PFC but not NAc (Tian et al., 2012) and no changes (Chao et al., 2014; Fragou et al., 2013) have been reported. In contrast, global methylation has been reported to be decreased in the NAc but not mPFC with cocaine self-administration (Wright et al., 2015). Opiates have been reported to cause a profound decrease in total genomic and repeat region DNA methylation in vitro (Knothe et al., 2016; Trivedi et al., 2014) and to a lesser extent in leukocytes from opiate treated subjects (Doehring et al., 2013). Here we extend these findings to heroin self-administration and DNA methylation levels across the mesolimbic circuit using multiple analytical methods. We observed neither global nor repeat element changes in methylation as a function of heroin self-administration or with environmental enrichment. Pyrosequencing of DNA repeat elements, whole genome bisulfite sequencing, and whole-genome ELISA all provided highly consistent data across the groups. While conceptually seductive, it is unlikely that large changes in genome-wide methylation would occur given that, as a prime regulation of gene-expression, de-methylation of the genome would cause dramatic dysregulation of gene expression. Rather than functioning as a blunt instrument, epigenetic changes are more likely to be locus- and temporally- specific, with some sites gaining methylation and others losing methylation. Further, it is well known that different regions of a given promoter region can increase or decrease the expression of a given gene depending on the transcriptional environment (Lemon and Tjian, 2000; Tropepe et al., 2006; Whitfield et al., 2012). These findings
demonstrate the need for site-specific methylation analyses that can be tied to gene expression analyses.

**Gene-Expression**

We have previously shown that enforced abstinence after heroin self-administration is accompanied by incubation of drug seeking during extinction testing and changes in mRNA expression in the brain (Kuntz-Melcavage *et al.*, 2009; Kuntz *et al.*, 2008a). Here, we extend these findings to show that environmental enrichment can attenuate relapse-like behaviors following a period of abstinence. Further, environmental enrichment appears to attenuate the expression of genes previously found to be increased following drug abstinence; a phenomena that appears to be independent of drug-taking history since both SH and EH heroin rats self-administered the same amount of drug. For example, basal levels of EGR1 in the mPFC of the enriched heroin group were significantly lower than the other conditions. Environmental enrichment has been previously shown to decrease basal levels of EGR1 due to the repeated exposure to novel objects and stimulating experiences (El Rawas *et al.*, 2009). Previous studies have also shown that inhibition of EGR1 expression prevents cue-induced cocaine seeking (Lee *et al.*, 2006). EGR2 follows a similar pattern of lower expression in the mPFC in the enriched heroin subjects compared to the other conditions. Decreased EGR2 expression has been linked with vaccine-induced attenuation of oxycodone self-administration in the brain (Pravetoni *et al.*, 2014). These findings suggest that decreases in mPFC EGR1 and EGR2 may contribute to the protective effect of enrichment on addiction-like behaviors such as motivation to work for drug, drug-seeking, and relapse-like behaviors (e.g., drug-induced reinstatement of heroin seeking).

Interestingly, EGR1 and EGR2 both show decreases in the NAc in the standard heroin, enriched saline, and enriched heroin conditions compared to standard saline subjects. This decrease
across the three conditions may result from the fact that subjects were exposed to rewards either in the form of heroin or in environmental enrichment. However, more testing is needed to evaluate the merits of this hypothesis. Of note, not all of the genes previously found (Kuntz-Melcavage et al., 2009; Kuntz et al., 2008a) to have been changed with abstinence were found to be altered here. Although the present study utilized a similar procedure as Kuntz et al. (2008) such as the same drug access period (3 h) and the length of drug abstinence (14 d)(Kuntz et al., 2008b), the present procedure also included a progressive ratio test and a more robust extinction and drug-primed reinstatement phases. These different parameters, along with the fact that sacrifice occurred 24 h later than in the previous studies and were done without anesthesia, could account for the inability to replicate earlier findings regarding changes in the expression of some genes.

**Site-Specific Differences in Cytosine Methylation**

DNA methylation was once perceived as a stable modification that persist for long periods of time. Recent advances in the field have shown that methylation status is more dynamic than previously thought (Schubeler, 2015), and can be altered with cocaine experience and reward learning (Day et al., 2013; Feng et al., 2015; Watson et al., 2015). Here, we extended these results and report that the availability of a reward can alter the methylation profile at specific regions of the genome. The EGR2 gene promoter region was found to be significantly hypomethylated in the mPFC in subjects that received either access to heroin or environmental enrichment compared with standard housed subjects that possessed no access to a reward. Closer inspection at the base resolution further showed a decrease in methylation status at promoter CG sites in the subjects exposed to heroin or enrichment, while CG sites in exon 2 show the opposite pattern: standard saline subjects have lower levels of CG methylation. Additionally, in the NAc, we found lower levels of methylation at
specific CG sites in the EGR1 gene in our standard saline group, compared to the other treatment
groups, which is associated with higher expression of this gene.

While there is much yet to be elucidated regarding the relationship between DNA
methylation and gene-expression, methylation of the promoter region is generally associated with
gene repression (Razin and Cedar, 1991). Here, we find that increased methylation of the EGR2
gene promoter is associated with increased gene-expression, whereas methylation of the gene body,
instead, is associated with increased gene-expression for both EGR2 and EGR1. Our findings add
to the growing body of literature that suggests that DNA methylation is not strictly a repressive
mechanism, but plays multiple roles in transcription regulation (for review, see (Jones, 2012)).
Moreover, the present data demonstrate the impact that the availability of a reward, either a drug
of abuse or environmental enrichment, can have on the methylation landscape in an individual.

To our knowledge, this is the first paper to quantify site-specific mCH in the context of
drug-abuse liability and environment. While mCH occurs at lower levels (most are 2-20% methylated) than mCG, it is enriched in neuronal cell types (occurring in approximately 2-6 % of
CNS cells) (Lister et al, 2013). Interestingly, mCH accumulates in the neuronal genome
concomitantly with synaptogenesis in the post-natal development of mouse and human. In this
study, we examined CH methylation in EGR1 and EGR2 in the mPFC and NAc and found two
sites to be differentially methylated between our 4 populations: site 1461 of EGR1 in the NAc and
site 569 of EGR2 in the NAc. There remains much to be learned in the field regarding the
relationship of CH methylation and fluctuations in gene expression; however, thus far it has been
shown to be a repressive mechanism whether in the 5’region, gene body or 3’ region (Guo et al,
2014). Here, we show that in the gene body of EGR1, decreased levels of CH methylation are
associated with increased gene expression in our standard saline group. Conversely, increased CH
methylation in the gene body of EGR2 was associated with increased gene expression in the
standard saline group. Just as CG methylation is not solely repressive, and may site-specifically
enhance gene-expression (Jones, 2012), CH methylation may augment gene-expression in a locus specific fashion. Taken together, these findings highlight the complex relationship of CH methylation and gene-expression.

We also show that basal methylation levels (as exemplified by our standard housed saline group) in the NAc and mPFC have remarkably similar CG methylation profiles in EGR1 and EGR2 promoters and gene bodies. This provides evidence for a common set point, regulated by undetermined mechanisms. Additionally, we observed extremely low levels in regions previously reported to dynamically change with reward learning (Day et al., 2013). Important distinctions between the Day et al. study and the present report are two: Day and colleagues examined a cue-food association and at early (not late) acquisition. Findings like those reported by Day et al., then, might be obtained were we to examine gene and epigenetic changes early (i.e., within the first 3 – 5 trials), rather than late, in acquisition. Also previous studies only used relative quantitation of methylation, the differences observed could potentially be shifts in background levels of methylation.

These findings also provide an example of how much more work is required to understand methylation patterns and their potential role in addiction. With the extensive efforts to understand the genetics of opiate addiction (Kreek et al., 2005; Levran et al., 2008), clearly a commensurate effort is justified to understand the epigenetic changes associated with opiate abuse and their role in addiction and relapse. Future studies will need to examine the epigenome in a cell-type specific manner to gain the clearest understanding.

**Conclusion**

The present study examined the effect of constant exposure to environmental enrichment on addiction-like behavior following heroin self-administration at the behavioral and molecular levels.
Constant exposure to environmental enrichment attenuated the motivation to work for heroin and reduced relapse-like behaviors following enforced abstinence. Global fluctuations in DNA methylation have been postulated to underlie addiction-like behavior. We found no evidence to support this, and maintain that the relationship between DNA methylation and behavior is most likely a function of locus-specific methylation in relevant genes. Environmental enrichment appeared to decrease the expression of some genes found to be altered due to heroin abstinence (e.g., EGR1). Additionally, heroin exposure and enrichment status appeared to influence the epigenetic landscape of specific sites in the genome. Future studies using platforms such as RNAseq should investigate other genes that are altered with enrichment status and thus confer resilience to drug-taking behaviors. Taken together, the data support that environmental enrichment can protect against addiction-like behaviors and provides further support for the use of alternative rewards to help protect and treat human addicts.
Chapter 7 Discussion

Addiction is a multifaceted individual and societal problem. As a disease of brain reward circuitry, addiction is characterized by 1) loss of control over drug taking; 2) drug seeking even when faced with negative consequences; 3) and devaluation of natural, alternative rewards that once brought pleasure. It is this last aspect which may be the most problematic for societies as a whole, as it results in consequences such as job loss, child neglect, strained relationships and stealing from friends or family members in order to buy more drug.

Over the past 20 years, there has been a rise in heroin use and addiction (Administration, 2013); most likely a result of the widespread use of opioids for pain management (Cicero et al, 2014; Peavy et al, 2012). Although opiates have a high addiction potential, not everyone who uses opiates will become addicted to them. In fact, rather like many people are able to drink socially and never have a problem limiting alcohol, only half of those that use heroin will succumb to addiction (SAMHSA, 2014). Interestingly, this phenomenon not only applies to humans. Animals will also demonstrate large individual differences when presented with the opportunity to self-administer drugs of abuse. Accordingly, it is very likely that individual behavioral responses have their root in molecular differences in brain regions that mediate the behavior. Based on this hypothesis, the goal of this thesis was to identify molecular differences in the reward pathway of animals who demonstrate resilience and vulnerability to addiction-like behavior. Figures 7A and 7B illustrate the goals and main findings of this dissertation.

Reward Devaluation and Heroin Self-Administration is Accompanied by Differential Reward Area Gene Expression

Chapters 2 and 4 made use of a rat model of reward devaluation and heroin self-administration developed by the Grigson laboratory. In short, an outbred group of Sprague-
Figure 7-1. – Representation of the experiments and findings featured in this dissertation.
Dawley rats are presented with the opportunity to ingest a sweet saccharin solution prior to the opportunity to self-administer heroin. As detailed in Imperio and Grigson (2016), rats in this paradigm will split into two behavioral phenotypes: those that prefer saccharin and take a small/moderate amount of heroin while escalating sweet intake (small saccharin suppressors), and those who develop an avoidance of the saccharin cue in favor of large amounts of heroin (large saccharin suppressors). The large suppressors not only demonstrate alternative reward devaluation and increased heroin taking, they also demonstrate increases in other addiction-like behaviors such as: shorter latencies to the first infusion, greater drug seeking when drug is not available, and they will work harder to earn a drug infusion (Imperio et al, 2015). In Chapter 2, we investigated the gene expression of the corticotropin releasing factor (CRF) pathway throughout reward pathway components. The extrahypothalamic CRF signaling pathway generates a site specific stress response across the brain. For example, in the hippocampus, CRF will enhance LTP (Chen et al, 2001) and memory formation (Refojo et al, 2011); in the VTA, the CRF receptors are located on both dopaminergic and non-dopaminergic neurons and activation through stress or ICV CRF infusion brings about increases in dopaminergic transmission (Dunn et al, 1987; Koob et al, 1999); and, in the mPFC, CRF increases anxiety-like behavior and activation of the HPA axis (Jaferi et al, 2006). In brief, we found that the large saccharin suppressor (large heroin taker) group demonstrated increased gene expression of CRF pathway components throughout major regions of the reward pathway: the hippocampus, mPFC and VTA. The similar profile of gene expression between these three brain regions is interesting given the role of these regions in reward learning. The hippocampus and mPFC, particularly, share a circuit that strongly influences associative learning (Li et al, 2015). Moreover, subsets of neurons in the mPFC and hippocampus have been shown to fire synchronously during learning, goal-directed behavior and REM sleep (Hyman et al, 2005; Li et al, 2015). It is possible that (presuming that increased CRF signaling mRNA expression is a reflection of CRF signaling protein expression) increased CRF tone may, in part, mediate
increased associative, and perhaps aversive learning between the saccharin cue and the imminent opportunity to self-administer heroin, and thus, bring about a faster or stronger cue-induced withdrawal effect. In support of this, Nyland and Grigson (2013) found that suppression of a cocaine-associated saccharin reward was accompanied by rises in circulating corticosterone (Gomez et al, 2000) much like what is observed in naloxone-precipitated withdrawal (Nyland et al, 2013).

Within the VTA, we found increased gene expression of CRFbp. As discussed in Chapter 2, CRFbp in the VTA has been found to increase (not blunt) CRF mediated potentiation of DA neurons (Ungless et al, 2003). The hippocampus and mPFC receive dense DA innervation from the VTA, and the VTA-mPFC-hippocampus dopaminergic signaling loop has been found to be integral for the persistence of aversive memories (Gonzalez et al, 2014; Rossato et al, 2009). Using a conditioned taste aversion (CTA) paradigm, Gonzalez et al. (2014) found that blockade of dopamine D1 receptors in the mPFC attenuated CTA and memory of footshock, while agonism of mPFC and hippocampal CA1 D1/D5 receptors enhanced the aversive memory persistence (Gonzalez et al, 2014). Given our findings, increased CRF signaling in the VTA leading to increased DA through the VTA-mPFC-hippocampus circuit may mediate the persistence of the associated aversive memory (acute withdrawal) of the heroin associated saccharin cue and may be a mechanism behind the drug-induced devaluation of an otherwise rewarding experience. Future studies should examine site-specific CRF and DA agonism and antagonism on drug-induced reward devaluation.

Lastly, Chapter 2 investigated the delivery of a CRFRI antagonist on drug-taking behavior in large and small suppressors. Systemic Antalarmin delivery prior to an extinction responding test (which assesses the strength or persistence of drug-seeking) diminished drug seeking in both large and small suppressors, although it did not change saccharin intake prior to the extinction session. It is also worth noting that the mitigating effects of Antalarmin were most pronounced in the large
suppressor group. As a future treatment strategy for drug addiction, this finding may highlight the need for better patient profiling in successful pharmacological interventions.

**Passive Infusion Does Not Recapitulate Self-Administration**

In Chapter 3, we investigated whether the CRF signaling gene expression changes observed in a self-administration model were a function of reward devaluation or self-administration behavior (or, perhaps, a combination of both) or a function of the varying doses inherent in a self-administration model. Using a passive infusion model, rats were again given the opportunity to ingest the saccharin cue prior to a passive injection of heroin. In this way, we were able to control for both self-administration behavior as well as a dose effect. As shown in Figure 3-1, 12 out of 20 animals will suppress saccharin intake when it precedes the passive delivery of heroin (large saccharin suppressors), while 8 animals had saccharin ingestion that is not different from those receiving saline injections (small saccharin suppressors). We then probed CRF pathway gene expression in the reward pathway components: hippocampus, mPFC, VTA, NAc and amygdala. We found that almost none of the molecular effects we observed in the self-administration model were present in the passive infusion model, with the exception that the CRFR2 gene was more highly expressed in the hippocampus of the large saccharin suppressor group (in Chapter 2, this was also observed). CRFbp was also more highly expressed in the large saccharin suppressor group, an effect that did not reach significance in the self-administration model. Moreover, no differential gene expression of the CRF pathway components was found in any other of the investigated reward regions. Several laboratories have reported differential molecular responses in animals participating in the self-administration of a drug versus animals receiving the drug passively (Radley et al, 2015) (Chen et al, 2008) (Greenwald et al, 2005). This should not be a surprising finding; self-administration involves a complex interplay of internal and external states.
including craving elicited by environmental associated cues, drug seeking, and even the motor aspects of self-administration. Moreover, just as a sense of loss of control over an aspect of one’s life is a stressful experience, animals who receive passive delivery of cocaine demonstrate stress responses such as avoidance of the cocaine associated chamber (Twining et al., 2009a) and rises in circulating CORT (Palamarchouk et al., 2009). Of course, several confounds in our study may be (at least in part) responsible for the observed disparity between SA and passive infusion, including: dose, route of administration and the length of the study. Future studies should examine reward area CRF pathway expression in models that would control for these variables, such as a yoked-infusion model.

**Environmental Enrichment, Heroin, and the Epigenome**

As stated above, one of the most insidious aspects of addiction is the devaluation of natural rewards. Not only is this behavior damaging to societies and families and the life of the addict, it may also serve as a barrier to addiction recovery. The availability of alternative rewards has been shown to increase the likelihood that human addicts will remain clean. Higgins et al., (1993) showed that addicts enrolled in a multifactorial behavioral treatment approach that included the opportunity to earn tokens toward patient-chosen enriching experiences and objects (such as ski passes, camera equipment, etc) with each cocaine-free urine specimen were more likely to remain abstinent than those who received only counseling (Higgins et al., 1993). Cason and Grigson (2013) showed that the opportunity to ingest sweet prior to the opportunity to self-administer cocaine was protective against drug-taking behavior in female rats (Cason and Grigson, 2013). In Chapters 2, 3 and 4, we explored reward pathway-specific gene expression in rats that displayed different preferences for an alternative reward (when paired with heroin). In Chapter 6, we investigated reward area gene
expression and regulation in rats that experienced another type of alternative reward: environmental enrichment.

As shown in Chapter 6, rats that are housed in an enriched environment demonstrate decreased drug seeking and motivation compared to rats housed in the standard condition. We, then, examined the gene expression of targets that had been previously shown to be changed with heroin self-administration (Kuntz-Melcavage et al., 2009; Kuntz et al., 2008a). The early response genes, EGR1 and EGR2 were both decreased in the mPFC of the enriched heroin group relative to the other 3 conditions (standard saline, enriched saline, standard heroin). Heroin has been shown to cause an increase of EGR1 expression in the cingulate cortex, NAc shell and core, and the caudate putamen while environmental enrichment has been shown to decrease basal levels of EGR1 in the cingulate cortex (El Rawas et al., 2009). Moreover, Lee et al. (2006) showed that blockade of EGR1 expression in the BLA decreases cue-induced cocaine seeking and reinstatement (Lee et al., 2006). Self-administration of cocaine is concurrent with increases in EGR2 in the mPFC and striatum (Gao et al., 2017) whereas decreased striatal EGR2 expression was found to be associated with decreased oxycodone self-administration in rats (an effect that was achieved by treatment with an oxycodone vaccine that limits the effect of oxycodone through bound antibodies) (Pravetoni et al., 2014). Taken together, enrichment may cause decreases in mPFC EGR1 and EGR2 gene expression, thereby providing a protective effect against drug-taking behavior.

Another important goal of Chapter 6 was to understand the mechanisms behind the observed gene expression changes seen with environmental enrichment. First, given the reports of global methylation changes accompanying the administration of various drugs of abuse (Trivedi et al., 2014), we performed multiple analyses of global methylation changes in the mPFC, NAc and VTA. Using LINE 1, SINE and ID methylation as a surrogate marker of global methylation, we found that neither heroin nor enrichment status caused changes in global methylation. 5mC ELISA showed no changes, as well. Additionally, there were no changes in methylation of genic
regions nor promoters across the genome. This was an expected finding, as adaptation to any internal or external environment at the level of gene expression (via DNA methylation) would presumably involve the upregulation of some genes and the downregulation of others. We then investigated the methylation status of the genes for which we observed differential gene expression (i.e., EGR1 and EGR2 – genes responsive to reward and drug administration, as discussed above). For NAc EGR1, we observed that the standard saline group had lower CG methylation levels than enriched saline, standard heroin and enriched heroin at sites across the second exon. This coincided with our gene expression results where standard saline had the highest gene expression of EGR1 relative to the other 3 groups. In the mPFC, we observed that, across the promoter region of EGR2, standard saline had the highest levels of methylation, yet the standard saline group also had the highest gene expression of EGR2. This finding highlights the complex role of cytosine methylation in gene expression regulation, and adds to the growing body of literature that indicates that cytosine methylation is not a strictly repressive mechanism (for review, see (Jones, 2012)).

Taken together, the results of the experiments in Chapter 6 indicate that environmental enrichment can attenuate aspects of drug-taking behavior – an effect that may be mediated by the expression and site-specific regulation of early response genes in key reward areas.

The Right and Left mPFC Demonstrate Differential Gene Expression

In Chapter 4, we explored the mPFC transcriptome of rats with different heroin-taking phenotypes. In confirming the observed gene expression changes with the orthogonal method of qRT-PCR, we discovered that the left and right mPFC do not always demonstrate the same levels of gene expression. This thought had not occurred to us previously, as we had followed the common practice of utilizing one hemisphere for one protocol and performing a different kind of analysis on the other (assuming that the two hemispheres were equivalent). When looking into the literature,
we discovered that the mPFC, especially, demonstrates several types of lateral specificity, including: structural differences and responsivity, connectedness to other brain areas, and roles in stress responsivity. In order to explore this further, we asked three questions: 1) What basal gene expression differences exist, if any, between the left and right mPFC? 2) Given the plethora of literature that speaks to laterality in the stress response, would the CRF pathway also demonstrate lateral differences? And finally, 3) Would the CRF pathway gene expression respond in a hemisphere-specific manner differently in a group of animals subjected to a behavioral paradigm (a passive heroin model that most likely included a stress component, and definitely included heroin, administration of which has been shown to augment the CRF system).

In Chapter 5, we hypothesized that a group of untreated (handled only) male rats would demonstrate several basal gene expression differences between the left and right mPFC. To do this, we employed RNA-Seq, a method that provides absolute quantitation of all genes expressed in a given area (or cell type). We found a surprising number of genes differentially expressed between the left and right mPFC, particularly those involved in pathways such as cell signaling and cell growth, development and proliferation and cellular assembly and maintenance. One particularly interesting finding was the increased expression of genes involved in insulin signaling: IGF2 and IGFBP2, as well as Sned1 (also known as Insulin responsive sequence DNA binding protein-1). Insulin signaling is known to be a regulator of mood and depression, perhaps through its enhancement of synaptic plasticity and adult neurogenesis. Insulin resistance and diabetes is associated with greater likelihood of depression (Musselman et al, 2003; Rasgon et al, 2003; Timonen et al, 2005). Moreover, improvements in depression symptoms is associated with increased insulin sensitivity (Hood et al, 2011), an effect that is linked to alterations in various members of the insulin-like growth factor family.

Given the reports of left/right mPFC dominance in individual resilience to stress as well as differential structural responses of the left and right mPFC to stress, we investigated whether the
CRF signaling pathway (a stress pathway) would exhibit hemisphere differences in response to the passive infusion paradigm featured in Chapter 3. Interestingly, in the hippocampus, we found that CRFbp in the left hemisphere was differentially expressed between saline, small suppressors and large suppressors. Moreover, the pattern of expression resembled a step-wise dose effect in that the greatest saccharin suppression coincided with highest expression of CRFbp (the reader will note that the dose of heroin was the same for small suppressors and large suppressors). We tested the strength of the correlation between saccharin suppression and CRFbp gene expression and found a significant correlation in the left hemisphere, but not the right. Taken together, these findings may provide evidence for differential gene expression responses, between the left and right hippocampus, to behavioral reactions such as drug-induced reward suppression. Future studies should examine the interaction of reward suppression and CRFbp expression in the left and right hippocampus in longer paradigms (which may bring about more exaggerated behavior and gene expression phenotypes) and through the use of pharmacological intervention. It should be noted that the gene expression studies reported in Chapter 2 were performed on an unknown mix of left and right hemispheres. In Chapter 2, we show that CRFbp in the hippocampus trends toward being higher in the large suppressor group (p=0.18), while in Chapter 3, we report that CRFbp is higher in the LS group; an effect that is driven by left hippocampal gene expression. Therefore, it is possible that significant differential expression of hippocampal CRFbp in the self-administration paradigm was obscured by the use of both left and right hemispheres.

Conclusions

As is shown in Figure 7-1, the aggregate findings of this dissertation provide a number of important findings and avenues for further research and clinical translation.
1) Clearly, the CRF system plays an important role in heroin self-administration (Chapters 2 and 3). First, alterations in gene expression within the CRF pathway are almost exclusively manifested in the SA model. Second, pretreatment with Antalarmin (a systemically bioavailable CRF antagonist) prior to a heroin self-administration session blunted heroin-seeking. Moreover, the large saccharin suppressors (large heroin-takers) exhibited a more profound response to Antalarmin treatment. This suggests that variable behavioral phenotypes may respond differently in clinical trials and should be considered in trial design.

2) This work continues a long history of our characterization of brain gene expression changes in response to substances of abuse (Freeman et al., 2002; Freeman et al., 2010; Kuntz-Melcavage et al., 2009; Kuntz et al., 2008a) Freeman and colleagues found that gene expression of the adenosine A2B receptor (Adora2b) was decreased in the mPFC of rats undergoing 10 days of enforced abstinence from cocaine self-administration (Freeman et al., 2010). In Chapter 4, we report that the gene expression adenosine A2A receptor (Adora2a) is elevated in the mPFC of rats who self-administered heroin (compared to saline controls). This finding adds to the body of literature implicating adenosine signaling in addiction-like behaviors (Justinova et al., 2003; Knapp et al., 2001; Shin et al., 2004). In Chapter 6 we extend the findings of Kuntz et al. (Kuntz et al., 2008a) and Freeman et al. (Freeman et al., 2010; Freeman et al., 2008) and show that the early response genes EGR1 and EGR2 are changed in response to both heroin self-administration and environment (which included the presence and absence of non-drug reward). In Chapter 2, we show that the gene expression of the Wnt signaling pathway gene, beta catenin, in the VTA is markedly elevated in small suppressors compared to large suppressors, while Freeman et al. and others found it to respond to cocaine self-administration (Freeman et al., 2010; Freeman et al., 2001b; Lynch et al., 2008) and Tacelosky et al. found that decreased
levels of wntless in the mPFC was associated with greater addiction-like behavior in heroin self-administering rats (Tacelosky et al, 2015). Moreover, in Chapters 2 and 6, we show that addiction-like behavior (Chapter 2, Antalarmin) and gene expression (Chapter 6, gene expression) persist even after periods of enforced abstinence. Taken together, the findings herein and those we have built upon add credence to the growing recognition that addiction and relapse liability are reflected by persistent changes in the brain.

3) The results of the environmental enrichment study (Chapter 6) provide a clear rationale for focusing on psychosocial support interventions in addition to potential pharmacotherapeutics. It has been long recognized that neither medication-assisted treatment (such as naloxone or methadone) nor twelve-step programs are optimal in and of themselves. Moreover, Chapters 2-4 and 6 suggest that there will be subsets of patients that respond most efficaciously to different approaches.

4) Finally, work described in Chapter 5 (laterality differences in gene expression), as well as Chapter 3 (lateral differences in the CRF pathway) provide important guidance for studies in neuroscience. Clearly, designs that execute gene expression studies in one hemisphere with confirmation in the same region of the other hemisphere should be interpreted with caution.

In closing, these studies provide a coordinated evaluation of gene-expression, addiction behavior and environmental influence. We provide further evidence that addiction is not a moral failing, but is 1) strongly influenced by brain changes and that 2) individual responses to drugs of abuse find their root in individual neurochemistries and different environmental circumstances. Further, we show that consideration should be given to laterality when examining neuromolecular phenomena. Lastly, we hold that the successful treatment of addiction will involve not condemnation and punishment, but holistic and personalized approach.
References

Administration SAaMHS (2013). Results from the 2012 national survey on drug use and health: summary of national findings. . Rockville, MD: Substance Abuse and Mental Health Services Administration.


associated with METH-induced increased H4K5Ac binding in the rat striatum. *BMC Genomics* 14: 545.


Cason AM, Grigson PS (2013). Prior access to a sweet is more protective against cocaine self-administration in female rats than in male rats. *Physiol Behav* 112-113: 96-103.


Chen Y, Brunson KL, Muller MB, Cariaga W, Baram TZ (2000). Immunocytochemical distribution of corticotropin-releasing hormone receptor type-1 (CRF(1))-like immunoreactivity


Lalumiere RT, McGaugh JL (2005). Memory enhancement induced by post-training intrabasolateral amygdala infusions of beta-adrenergic or muscarinic agonists requires activation
of dopamine receptors: Involvement of right, but not left, basolateral amygdala. Learn Mem 12(5): 527-532.


U.S. Dept of Health and Human Services SAaMHSA, Center for Behavioral Health Statistics and Quality (2012). Results from the 2012 National Survey on Drug Use and Health: Summary of National Findings.


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