REGULATION OF CIRCADIAN GENES BY COCAINE IN STRIATAL REGIONS AND THEIR ROLE IN DRUG REWARD

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DEDICATION

First of all, I would like to dedicate this body of work to my parents, Félix A. Falcón-Torres and Carmen M. Morales-Ortiz. Your unconditional love and support throughout the years have led me to be what I am today. It is because of you that I have followed my dreams and enjoyed life's adventures to the fullest. You have instilled in me a great appreciation for family values, a sense of tolerance, and a devotion to hard work that have helped me throughout my graduate school years. I would also like to dedicate this to my brothers Felo, Carlos and Luiggie. Thanks for showing me what true brotherly love is: Love, acceptance, compassion and unconditional support through good times and bad.

Additionally, I would like to dedicate this work to a person that was and will always be my best friend, Antonio Morales-Guzmán. Dear grandpa, even though you're not here anymore, you will always be with me in my thoughts. I will never forget how much fun we used to have talking about politics, history, science, and social issues. You always supported me in every endeavor and provided guidance and constructive criticism whenever I needed it.

To the rest of my family and friends, this is for you.

REGULATION OF CIRCADIAN GENES BY COCAINE IN STRIATAL REGIONS AND THEIR ROLE IN DRUG REWARD

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REGULATION OF CIRCADIAN GENES BY COCAINE IN STRIATAL REGIONS AND THEIR ROLE IN DRUG REWARD

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Disruptions in circadian rhythms are associated with neuropsychiatric disorders, including drug addiction. Indeed, mounting evidence reveals a role for the circadian clock in the regulation of drug reward and reward-related behaviors. Conversely, drugs of abuse are known to dysregulate circadian-associated processes and entrain locomotor behavior. The circadian clock is governed by a master pacemaker in the Suprachiasmatic Nucleus(SCN) of the anterior hypothalamus. However, the components of this circadian clock machinery are expressed throughout the brain and body, allowing for the occurrence of SCN-

dependent or independent peripheral oscillators. One such brain circuit in which circadian genes are expressed is the mesolimbic dopaminergic pathway, containing brain regions such as the Nucleus Accumbens(NAc) and the Caudate Putamen (CP), among others. We set out to investigate how repeated cocaine exposure regulates core clock circadian genes in striatal regions and conversely, how core clock circadian genes regulate cocaine's rewarding effects. Additionally, the potential regulation of rhythmic dopamine receptor expression directly by clock components and how their rhythmic expression is altered by repeated cocaine exposure was assessed. Chapter 3 determined circadian gene regulation in both the NAc and CP by cocaine. Not only did chronic cocaine upregulated a number of circadian genes at a specific timepoint, like *Npas2* and the *Per* genes, but also altered or disrupted 24-hr rhythmic expression of these genes. Chapter 4 investigated the role of core circadian clock genes in cocaine reward, as measured by conditioned place preference. Npas2 mutant mice exhibited a decreased preference for cocaine, an effect that was recapitulated by viral-mediated *Npas2* knockdown specifically in the NAc. Per mutant mice displayed an increase in cocaine preference. Knockdown of *mPer1* and *mPer2* in the NAc led to a trend towards increased preference. Chapter 5 identified a potential role for NPAS2 in the regulation of dopamine receptor rhythmic expression in the NAc, and that chronic cocaine disrupts this rhythmicity. These findings suggest an important role for Npas2 as mediator of cocaine responses in the NAc. Moreover, they further elucidate the bidirectional interactions between

the circadian and reward systems, implicating the circadian control of the dopaminergic system in this interplay.

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LIST OF DEFINITIONS

3’- UTR – 3’- Untranslated Region

AAV – Adeno-Associated Virus

ANOVA – Analysis of Variance

Bmal1 – Brain and Muscle ARNT-like 2

cDNA – complementary Deoxyribonucleic Acid

ChIP – Chromatin Immunoprecipitation

CK1 – Casein Kinase 1

CLOCK – Circadian Locomotor Output Cycles Kaput

CP – Caudate Putamen

CPP – Conditioned Place Preference

CREB – cyclic AMP Response Element Binding Protein

Cry 1, 2 – Cryptochrome 1, 2

DAPI - 4',6-diamidino-2-phenylindole

DAT – Dopamine Transporter

DRD1 – Dopamine Receptor D1

DRD2 – Dopamine Receptor D2

DRD3 – Dopamine Receptor D3

EDTA – Ethylenediaminetetraacetic Acid

EGFP – Enhanced Green Fluorescent Protein

ERK – Extracellular signal-Regulated Kinase

GABA – Gamma Aminobutyric Acid

Gapdh – Glyceraldehide-3-Phosphate Dehydrogenase

GFP – Green Fluorescent Protein

Glu – Glutamate

GSK3β – Glycogen Synthase Kinase 3 beta

IHC – Immunohistochemistry

MAOA – Monoamine Oxidase A

MPON – Medial Preoptic Nucleus

NAc – Nucleus Accumbens

NPAS2 – Neuronal PAS domain protein 2

PA – Positive Affect

PCR – Polymerase Chain Reaction

P-CREB – Phosphorylated –CREB

Per 1, 2, 3 – Period 1, 2, 3

PVDF - Polyvinylidene difluoride

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcriptase-PCR

SCN – Suprachiasmatic Nucleus

SDS – Sodium Dodecyl Sulfate

shRNA- short hairpin RNA

SERT – Serotonin Transporter

SSRI – Selective Serotonin Reuptake Inhibitor

TH- Tyrosine Hydroxylase

TPH – Tryptophan Hydroxylase

VTA- Ventral Tegmental Area

CHAPTER ONE:

DRUG ADDICTION AND THE CIRCADIAN SYSTEM

Drug Addiction: Basics

Drug addiction is a devastating chronic relapsing disease that affects millions of people worldwide and contributes to the death of over 500,000 Americans per year (NIDA, 2010). In fact, retrospective comparative studies between 1990 and 2000 have found that when combining the number of deaths attributed to both legal and illicit drugs, substance use and abuse disorders become the leading cause of actual deaths in the United States (Mokdad et al., 2004). Moreover, the effect that addiction has on society is overwhelming considering the combined medical, economic, criminal, and social impact, which amounts to over half a trillion dollars a year in the US alone (NIDA, 2010). The clinical picture of addiction is marked by compulsive drug use that the individual cannot fully control despite adverse consequences. The Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) establishes several criteria to be met in order to be diagnosed as substance dependent (Figure 1.1). These include development of tolerance, manifestation of a withdrawal syndrome for the substance or continued use of the substance to remedy withdrawal symptoms, increase or escalation of drug intake, and becoming preoccupied or devoting a great deal of time in obtaining the drug despite persistent physical, psychological and social problems (American Psychiatric Association DSM-IV, 2004).

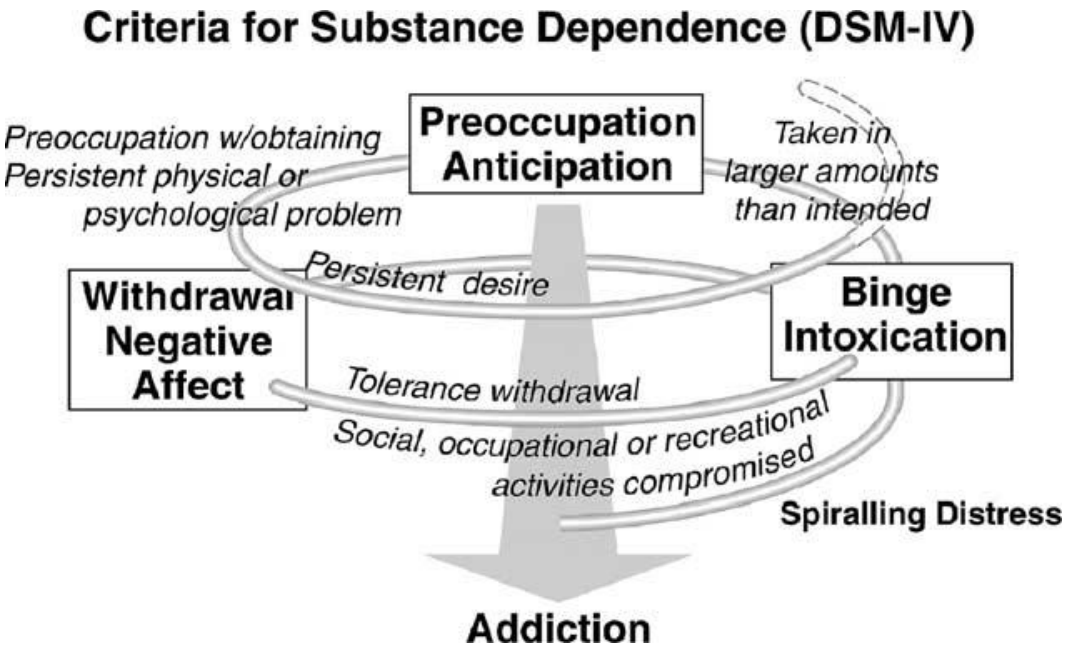

Figure 1.1 Criteria for Substance Dependence (DSM-IV). Diagram describing the spiraling addiction cycle showing its three major components (Preoccupation/Anticipation, Binge/Intoxication, and Withdrawal/Negative affect) along with the different criteria for substance dependence as described in the DSM-IV. Drug addiction is a chronically relapsing disorder that is characterized by a compulsion to seek and take drug, loss of control in limiting intake, and emergence of a negative emergence state when access to the drug is prevented. Adapted from Koob et al., 2004.

Recent studies have revealed that this transition towards compulsivity is likely a pathology of brain neuroplasticity (Kalivas and O'Brien, 2008). Repeated exposure to drugs of abuse leads to long-lasting changes in key neuronal circuitry and specific brain regions that are not easily reversed. Some of these regions include the Nucleus Accumbens (NAc) and the Ventral Tegmental Area (VTA), both of which are part of the mesolimbic dopaminergic system and play a role in reward-related processes (Figure 1.2; Hyman et al., 2006). The mesolimbic dopaminergic system has been viewed as the common circuitry shared by all drugs of abuse in terms for their acute rewarding effects (Nestler, 2005). Extensive research has established that several additional brain regions are also involved in the acute rewarding effects and in the chronic effects of drugs of abuse, as well as natural rewards (Nestler, 2005; Koob and Volkow, 2009). These include the amygdala, hippocampus, hypothalamus, and regions of the frontal cortex, among others (reviewed by Koob and Volkow, 2009). Although many aspects of drug addiction have been studied, there is still no truly effective treatment for this chronic disease. A high probability of relapse, regardless of the length of abstinence, makes treatment and recovery very challenging. Understanding the molecular mechanisms that underlie the pathophysiological abnormalities that lead from recreational drug use to addiction may help in designing new and more effective treatments.

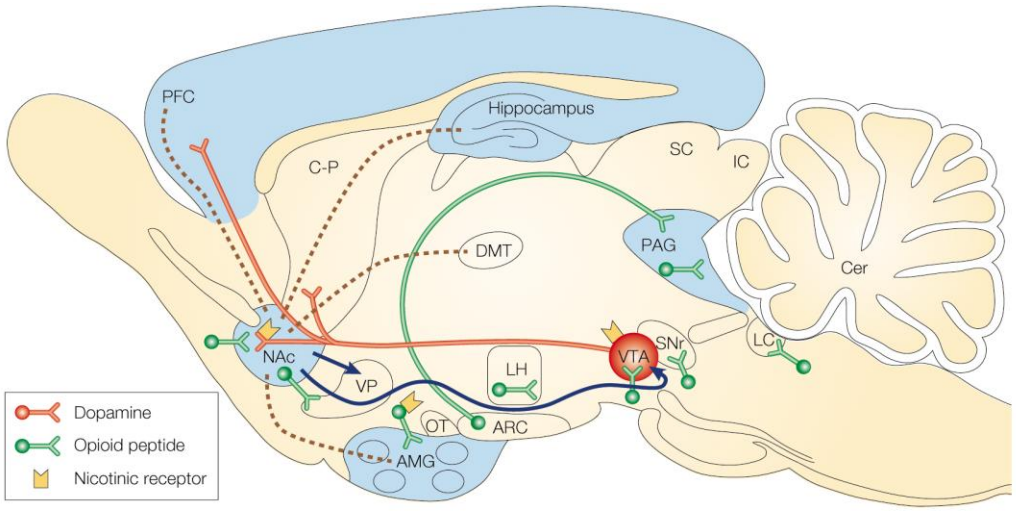

Figure 1.2 Diagram depicting the mesolimbic dopaminergic pathway in red. This VTA-NAc projection is important in the acute rewarding effects of drugs of abuse. Dopamine levels are increased in the NAc in response to drugs of abuse. Other neurotransmitters and neuropeptides, like glutamate, opioids, and orexin, also play a role in the modulation of these neuronal circuits involved in addiction. Circadian genes are also expressed in all of these reward-related regions and are thought to contribute to the development and maintenance of addictive behaviors. Adapted from Nestler, 2001.

Co-morbidity between addiction/substance use disorders and other psychiatric disorders

The majority of the brain regions that are involved in drug addiction are also involved in other neuropsychiatric disorders, like mood disorders and anxiety. Indeed, there is a high degree of comorbidity between substance abuse and primary psychiatric disorders including schizophrenia, mood and anxiety disorders, and attention-deficit hyperactivity disorder (Hasin et al., 2007; Kessler et al., 2005). Among individuals with schizophrenia, 40% to 60% abuse drugs or alcohol, and over 90% smoke cigarettes (George et al., 2002). Alcohol dependence has shown significant associations with bipolar I, bipolar II, specific phobias, and histrionic and antisocial personality disorders (Hasin et al., 2007). Bipolar disorder has a higher prevalence of substance-use disorders than any other psychiatric illness (Swann, 2010). In fact, alcohol or substance abuse was reported to precede the first manic episode in about one-third of bipolar patients (Swann, 2010). Cocaine use has also been reported to associate with several psychiatric disorders, like antisocial personality disorder, major depressive disorder, posttraumatic stress disorder and bipolar disorder (Falck et al., 2004; Brown, 2005). Indeed, about 20% of patients in treatment for cocaine use disorder also have an affective disorder (Carroll et al., 1997). Interestingly, severity of cocaine dependence has also been associated with psychiatric and substance use disorder comorbidity. Again, bipolar disorder and antisocial personality disorder were the ones associated with past heavy cocaine use (Ford et al., 2009). Common genetic and other neurobiological factors may be

responsible for the high comorbidity between these psychiatric disorders. Drug addiction may be more prevalent in individuals with a compromised circadian clock, or with mood disorders which may have a circadian basis, such as Major Depressive Disorder, Bipolar Disorder, and Seasonal Affective Disorder, among others mentioned above (Kandel et al., 2001; Grandin et al., 2006; McClung, 2007). Thus, dysfunction of the circadian clock and disruption of circadian rhythms is of increasing interest in the study of neuropsychiatric disorders.

Circadian Rhythms and the molecular clock

Most living organisms exhibit daily cycles in behavior and physiology that enable them to adapt to their environment and react to a variety of stimuli known as Zeitgebers or "time-givers" (e.g. light, food, etc.). In mammals, the central pacemaker that controls most of these activity rhythms is located in the Suprachiasmatic Nuclei (SCN) of the anterior hypothalamus and is primarily entrained by light (Reppert and Weaver, 2001). In turn, the master clock in the SCN coordinates the timing and activity of other oscillators in other areas of the brain and in peripheral organs, like the kidney and liver (Reppert and Weaver, 2002). Thus, circadian clocks are present throughout the body and regulate a plethora of metabolic and behavioral rhythms. The molecular mechanisms that underlie the circadian clock have been conserved throughout evolution, from cyanobacteria and fungi to insects and mammals.

The circadian clock (Figure 1.3) is based on a series of interconnected transcriptional positive-negative feedback loops that are regulated over the

course of twenty-four hours in the absence of environmental input (Reppert and Weaver, 2001; Ko and Takahashi, 2006). In mammals, the circadian locomotor cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL-1) proteins act as major transcriptional activators by forming a heterodimer that promotes transcription of the *Period* genes (*Per1, Per2,* and *Per3)*, the *Cryptochrome* genes *(Cry1* and *Cry2)*, as well as many other genes by binding to E-box elements (CANNTG) in their promoters (Reppert and Weaver, 2001). Following translation of the PER and CRY proteins, they are phosphorylated by casein kinase 1 (CK1) ε and δ, and glycogen synthase kinase 3β (GSK3β). These phosphorylation events can alter PER and CRY stability, dimerization, and nuclear entry (Harms et al., 2003). The PER and CRY proteins dimerize and enter the nucleus to inhibit CLOCK-BMAL1 mediated transcription, hence creating a negative feedback loop. An adjoining oscillatory feedback loop that regulates the expression of *Bmal1* by binding to RORE elements in its promoter is composed of the nuclear receptor REV-ERBα and the transcriptional regulator RORA (Reppert and Weaver, 2001). In forebrain regions or in conditions where CLOCK is nonfunctional, Neuronal PAS domain protein 2 (NPAS2), a protein similar in structure and function to CLOCK, can induce expression of the *Per* and *Cry* genes (Reick et al., 2001; Debruyne et al., 2006). Interestingly, NPAS2, which has high expression in striatal regions, has been linked to the formation of emotional memory, sleep, and food entrainment (Garcia et al., 2000; Dudley et al., 2003; Franken et al., 2006).

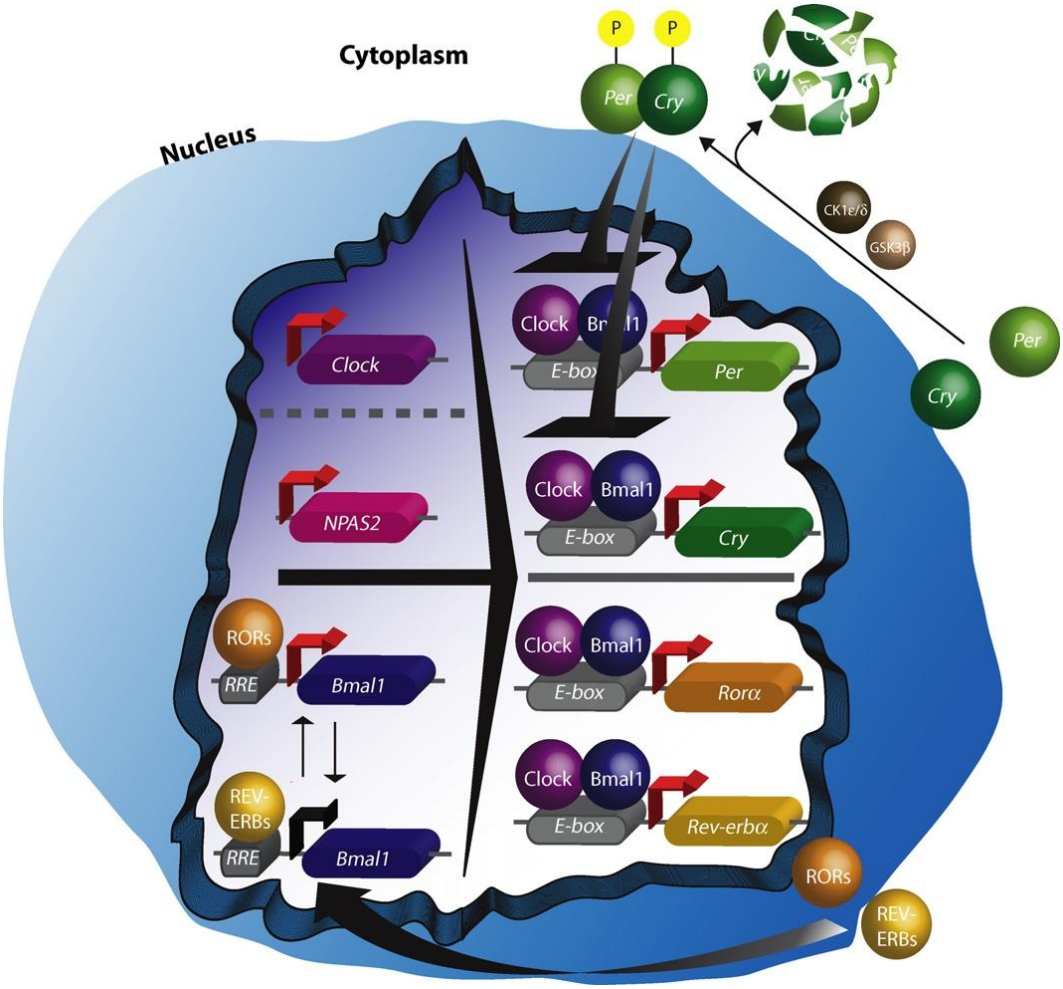

Figure 1.3 Cartoon depicting the molecular clock. CLOCK and BMAL1 (or NPAS2 and BMAL1) regulate the expression of the *Period* and *Cryptochrome* genes. These are translated in the cytoplasm and are phosphorylated. They enter the nucleus and inhibit the activity of CLOCK:BMAL1. A separate loop depicted at the bottom of the nucleus shows the regulation of *Bmal1* by Rorα and Rev-erbα. Adapted from Falcon and McClung, 2009.

A major target of the master circadian clock is the pineal gland, which results in periodic discharge of the hormone melatonin. This hormone is exclusively released at night, even in nocturnal animals, and has been found to promote and regulate sleep and other rhythmic physiological events including seasonal adaptations (Pandi-Perumal et al., 2006).

Diurnal and circadian rhythms in addictive processes

Drug addiction has long been linked to disruptions in diurnal rhythms. For example, drug addicts generally have severe disruptions in their sleep/wake cycle, activity cycles, eating habits, as well as, abnormal rhythms in body temperature, hormone levels, and blood pressure (Wasielewsky et al., 2001; Jones et al., 2003). The disruptions in sleep following drug use are highly problematic, persist long after drug use has ceased, and very often lead to relapse (Jones et al., 2003). Many of these disruptions were originally thought to arise as an indirect result of chronic exposure to drugs of abuse; however, studies have shown that repeated drug use can directly affect ongoing diurnal rhythms. For example, cocaine exposure was found to alter the rhythms of autonomic, immune and sleep mechanisms (Irwin et al., 2007; Morgan et al., 2006). Human studies have found that activation of the reward system, experienced as the mood state positive affect (PA), also follow diurnal and circadian rhythms (Murray et al., 2009). Studies found that under naturalistic conditions, or a regular schedule, PA exhibited a diurnal rhythm that peaked in

the afternoon. Moreover, a second study done under a constant routine procedure, where light and temperature were controlled and kept constant still revealed a circadian rhythm in PA that synchronized with core body temperature rhythms (Murray et al., 2009). There is also a diurnal variation in the sensitivity and pharmacological responsiveness to almost all drugs of abuse (Manev and Uz, 2009). Indeed, retrospective studies analyzing the admission of drug overdose patients in the emergency room of urban hospitals revealed that the majority of patients presented at around 6:30 pm compared to other times of day, suggesting a diurnal effect (Raymond et al., 1992), though there may be environmental and societal factors that influence this time of day effect as well. The use of addictive drugs has been found to follow seasonal patterns, with an increase in cocaine and alcohol use predominantly during the winter, when individuals are more susceptible to depression (Satel and Gawin, 1989; McGrath and Yahia, 1993). In addition, people with genetic sleep disorders and insomnia are more prone to addiction (Shibley et al., 2008).

Drug sensitivity is associated with rhythm abnormalities in animal models as well. Rats that were selectively bred based on a high preference for ethanol versus a low preference for ethanol have a shorter free-running period when animals are housed in constant light. One of the lines (the HAD line) also display a “splitting” of circadian activity in that they show two distinct bouts of activity in constant light which is not seen in the low ethanol preferring lines (Rosenwasser et al., 2005). A modest shortening of the free-running period was also found in ethanol-preferring mice compared to those selectively bred for low ethanol

preference (Hofstetter et al., 2003). These results suggest that genetic ethanol preference is associated with abnormal circadian rhythms.

Several animal studies of addiction have shown that there are diurnal differences in drug-induced behavioral responses, specifically locomotor activity, drug sensitivity, sensitization, conditioned place preference (CPP), and self-administration. A study by Baird and Gauvin found that rats display an increase in the sensitivity to the reinforcing properties of cocaine at 1:00am and 1:00pm compared to rats tested at 7:00am and 7:00pm, indicated by self-administration at lower doses and decreased drug intake (Baird and Gauvin, 2000). However, in general rats show a striking diurnal pattern of self-administration under a discrete trial procedure (DT3, three trials/hr) with a greater intake during the active dark phase than during the light phase (Lynch et al., 2008; Roberts et al., 2002). Interestingly, cocaine intake is significantly increased and the diurnal pattern of intake is nearly abolished when animals are given high doses of cocaine (2.5 mg/kg) or access to more trials (Roberts et al., 2002). This loss of diurnal intake rhythms may be very important in the development of addiction in which there is a loss of control and escalation of drug intake that interferes with normal activity (Ahmed and Koob, 1998). Following up on these studies, the same group found that in rats that were kept under constant dim-light (<2 lux) conditions, cocaine self-administration free-runs with period length (tau) of ~24 hrs. When the rats were housed under constant light conditions (1000 lux), the free-running rhythm in cocaine self-administration lengthened to a tau of ~26 hrs (Bass et al., 2010). Thus, the pattern of cocaine intake observed is circadian and

seems to be regulated by an endogenous oscillator. In contrast to the self-administration studies, mice treated for several days with cocaine during the day show a greater level of sensitization than those treated at night (Akhirasoglu et al., 2004; Abarca et al., 2002). Moreover, conditioned place preference for cocaine also displays a diurnal rhythm, with greater effects seen when drug is given during the day than during the night (Kurtuncu et al., 2004; Abarca et al., 2002). Conversely, studies performed in rats revealed that in opposition to short-term sensitization, long-term sensitization (2 weeks after last injection) was greater when the drug was given at the onset of the dark phase (Sleipness et al., 2005). These studies suggest that there is a change in the reward value for the drug and locomotor sensitivity to the drug over the light/dark cycle that is still not well understood. Indeed, differences in the light/dark cycle have been found to influence drug reinstatement (Sorg et al., 2011). Rats that were exposed to photoperiod shifts to shorter day lengths showed a suppression of drug-induced reinstatement of CPP when compared to a group exposed to shifts to longer day lengths. Hence, photoperiod seems to modulate drug-induced reinstatement and could explain some of the seasonal differences observed in drug-related behaviors.

A study by Sleipness et al. (2007) found that the SCN plays a role in the diurnal regulation of cocaine reward-related behavior. In this study, the authors found that acquisition of CPP behavior was tonically influenced by the SCN, as extinction of CPP behavior was SCN-dependent and reinstatement of CPP behavior was SCN-independent, suggesting an extra-SCN oscillator at work in

mediating this behavior (Sleipness et al., 2007a). Many of these diurnal differences in models of addiction may be due to diurnal regulation of dopaminergic transmission in the mesolimbic pathway. In fact, rhythms of cocaine sensitivity correlate with rhythms in postsynaptic levels of dopamine and the activity of the dopaminergic receptors in striatal regions (Naber et al., 1980). Interestingly, studies in *Drosophila* also found that dopamine receptor responsiveness displays a diurnal modulation (Andretic and Hirsh, 2000). Additionally, in mammals the expression of nearly all of the elements involved in dopaminergic transmission have a diurnal rhythm, including the dopamine receptors, the dopamine transporter, and tyrosine hydroxylase (Weber et al., 2004; Schade et al., 1995; Shieh et al., 1997). These diurnal differences in dopamine transporter and tyrosine hydroxylase expression levels are somewhat SCN-dependent, since SCN-lesioned animals have dampened rhythms in comparison to sham controls (Sleipness et al., 2007b). Moreover, a recent study by Hampp et al. (2008) found that the monoamine oxidase A (MAOA) gene which metabolizes dopamine, is a transcriptional target of BMAL1 and the PER2 protein. PER2 positively regulates its expression and mice with a mutation in *Per2* (*Per2*^{Brdm1}) have a decrease in *Maoa* expression in the NAc and VTA. These mice also have an increase in midbrain dopamine levels and release and an increase in the sensitization to cocaine (Hampp et al., 2008; Abarca et al., 2002).

In response to drugs of abuse, mesocorticolimbic dopaminergic activity leads to long lasting plasticity in the glutamatergic projections from the prefrontal

cortex to the primarily GABAergic NAc neurons (Kalivas, 2007). This altered plasticity is thought to be very important in the development of addiction (Kalivas, 2007). Extracellular levels of glutamate (Glu) and gamma-aminobutyric acid (GABA) in the dorsal striatum and NAc have both a diurnal pattern in light/dark conditions and a circadian rhythm in constant conditions with highest levels at night (Castaneda et al., 2004). Perfusion with melatonin prevents the daytime decrease in both Glu and GABA levels thereby dampening the rhythm (Marquez de Prado et al., 2000). This suggests that melatonin regulates striatal rhythms in Glu and GABA transmission. Moreover, expression of the vesicular glutamate transporter 1 (VGlut1) protein in synaptic vesicles has a diurnal rhythm with high levels at the start of the light period which decline by noon, rise again at the star of the dark period and fall again at midnight (Yelamanchili et al., 2006). Mice lacking *Per2* do not have any rhythm in *VGlut1* expression, suggesting that components of the circadian clock regulate glutamatergic vesicular sorting. Furthermore, mice with a mutation in *Per2* also show an increase in glutamate levels on the extracellular space of the NAc due in part to a reduction in levels of the glutamate transporter *Eaat1*, and this increase in glutamate is involved in the increased alcohol intake measured in these mice (Spanagel et al., 2005). Thus *Per2* plays an important role in regulating the expression of key genes involved in glutamatergic transmission in the striatum.

Circadian genes in animal models of drug addiction

Animal studies of drug responsiveness, sensitization, and reward have found that the circadian genes are important regulators of the behavioral responses to drugs of abuse. The first studies that revealed this relationship were done in *Drosophila*, and found that flies with mutations in the circadian genes *Per, Clock, Cycle,* or *Doubletime* all fail to sensitize to cocaine following repeated exposure, while those that had a mutation in the *Timeless* gene showed normal cocaine responses (Andretic et al., 1999). These pioneering studies provided evidence for the impact of specific circadian genes on addictive processes, which might be conserved throughout evolution. Following these studies, several groups found that cocaine is able to induce or repress specific circadian gene expression in various regions of the mammalian brain. Yuferov *et al.* found that *rPer1* was induced in the dorsal striatum following acute cocaine, while *rPer2* was only induced following a chronic "binge" pattern of cocaine (Yuferov et al., 2003). Furthermore, Uz *et al*., found that chronic cocaine treatment (rather than acute in most cases) resulted in the up or downregulation of several circadian genes in both the striatum and hippocampus (Uz et al., 2005a). These changes were distinct from those observed after chronic treatment with the antidepressant, fluoxetine. Moreover, a recent microarray study in animals self-administering cocaine found that with one day of withdrawal, 29 genes were differentially regulated in the dorsal striatum that are known to have a circadian function or be associated with the circadian system (Lynch et al., 2008). Using pathway analysis software, Lynch et al. found that

indeed changes to the circadian system in the dorsal striatum represented the most significantly altered pathway following cocaine self-administration (Lynch et al., 2008). These results suggest that alterations in the molecular clock in the striatum are important in a relevant model of addiction.

The importance of the circadian genes in cocaine preference was first shown by Abarca et al., who found that mice that lack a functional *mPer1* gene failed to sensitize to cocaine and show a complete lack of cocaine reward as measured by CPP. In contrast, mice that lack *mPer2* exhibited a hypersensitized response after repeated drug exposure with no change in cocaine-induced place preference (Abarca et al., 2002). In addition, *mPer1* may partially regulate morphine dependence, since mice treated with a DNAzyme towards this gene and morphine simultaneously show a reduction in the conditioned preference for the drug, while those that were treated with the DNAzyme after the morphine treatment did not show a difference when compared to the control group (Liu et al., 2005). This regulation of morphine reward by *mPer1* could be through its regulation of extracellular signal-regulated kinase (ERK) signaling since targeted disruption of *mPer1* by DNAzyme prevents the increase in ERK expression that is seen following morphine treatment (Liu et al., 2007). Further studies have found that *mPer2* is involved in influencing alcohol consumption. Spanagel and colleagues found that mice carrying a mutation in the PAS domain of *Per2* have an increase in alcohol intake that is linked to changes in glutamatergic transmission (Spanagel et al., 2005). The authors also found that variations in the *Per2* gene in humans are linked to modulation of alcohol intake, making

these variations functionally relevant to human addiction. Mice with a mutation in the *Clock* gene (Δ19) show a robust sensitization to cocaine, an increase in cocaine preference, and an increase in the reward value for cocaine as measured by intracranial self-stimulation following cocaine treatment (McClung et al., 2005; Roybal et al., 2007). These mice also have an increase in dopaminergic activity in the VTA which may be responsible for the increase in reward value for cocaine (McClung et al., 2005). This includes an increase in dopamine cell firing, bursting, and levels of TH and phosphor-TH (McClung et al., 2005). In further studies it was found that these mice display a complete behavioral profile that is very similar to human bipolar patients in the manic state (Roybal et al., 2007). Interestingly, mania is very often associated with an increase in psychostimulant use (Brown, 2005).

Over the last decade, there has been increasing evidence about the impact or influence that circadian rhythms might play in addictive processes (Figure 1.4). Disruptions of the circadian system have been shown to regulate responses to drugs of abuse and can modulate reward-related learning. Conversely, drug use, or reward presentation, seem capable of influencing circadian timing. This thesis work focuses on how repeated cocaine exposure regulates circadian genes and their rhythmic expression in striatal regions, while also investigating the role of these circadian genes in behavioral responses to cocaine. Additionally, in order to better understand how diurnal differences in behaviors arise or are disrupted by cocaine, rhythms of dopaminergic receptors

in the NAc were assessed before and after cocaine treatment, as well as, how these receptors might be regulated by components of the circadian machinery.

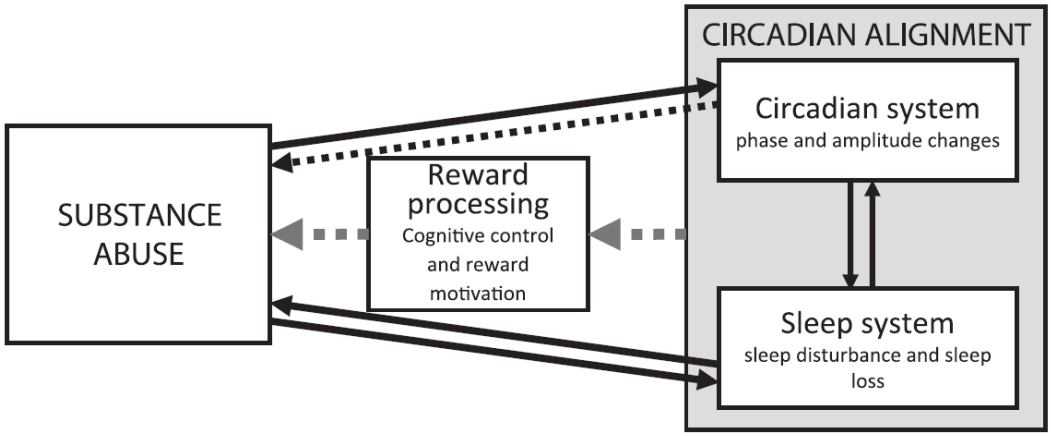

Figure 1.4 Schematic depiction of the bidirectional interaction between addiction/substance abuse and the circadian system. Circadian modulation of reward might be mediated by direct or indirect projections from the SCN or by circadian oscillators in reward-related regions (hatched arrows). An extensive body of literature has shown the effects of substance abuse on circadian and sleep parameters (solid lines). Adapted from Hasler et al., 2012.

CHAPTER TWO

BASIC INTRODUCTION OF METHODOLOGY AND TECHNIQUES

Behavioral Techniques

The work in this thesis made use of conditioned place preference and locomotor assays as tools to determine the effects of various genetic manipulations on mice behavior and on the rewarding properties of cocaine. Mice undergoing these behavioral tests were either mutant or knockout animals for specific circadian genes, or were infused with viral vectors expressing a short hairpin sequence specific for the circadian gene in question in order to knock down its expression via RNA intereference (RNAi) mechanisms.

Conditioned Place Preference (CPP)

The conditioned place preference procedure is a standard preclinical behavioral model widely used in rodents to study the rewarding and aversive properties of drugs (Prus et al., 2009). CPP is less time consuming than other more complex techniques to study drug reward and drug seeking behaviors, like the self-administration paradigm. It provides, however, an indirect assessment of a drug's or substance's rewarding potential based on classical conditioning. The positive rewarding properties of a substance are established by showing that animals prefer a context previously associated with that substance, compared to one associated with vehicle or saline. For this study, a three-chamber CPP box

was used (Figure 2.1a). The center compartment has no special characteristics and is only available on pretest and test days. The outer chambers of the CPP box differ in both visual (stripe versus solid color) and tactile (small grid vs large grid holes) cues. The protocol used in this thesis is based on an unbiased approach where the drug or vehicle is assigned to a compartment regardless of baseline preferences (Figure 2.1b). On day 1, mice were exposed to a 20 min pretest session to measure baseline preferences. This was followed by four daily consecutive conditioning sessions (days 2-5, 30 min each) and one last drug-free 20 min test session on day 6. The difference between the drug-paired side and the saline-paired side served as a reliable indicator of the rewarding effects of the drug. Several doses of cocaine were used, ranging from 2.5mg/kg to 10mg/kg.

Locomotor response to a novel environment

Locomotor behavior is a widely used behavioral technique to study the effects of pharmacological or genetic manipulations on an animal's baseline activity. In this study, locomotor behavior was measured to examine the effects of circadian gene manipulations on overall locomotor activity. Animals were placed in a home-cage environment inside a locomotor chamber with a photocell detection system to measure both ambulatory and fine movement for 2 hrs.

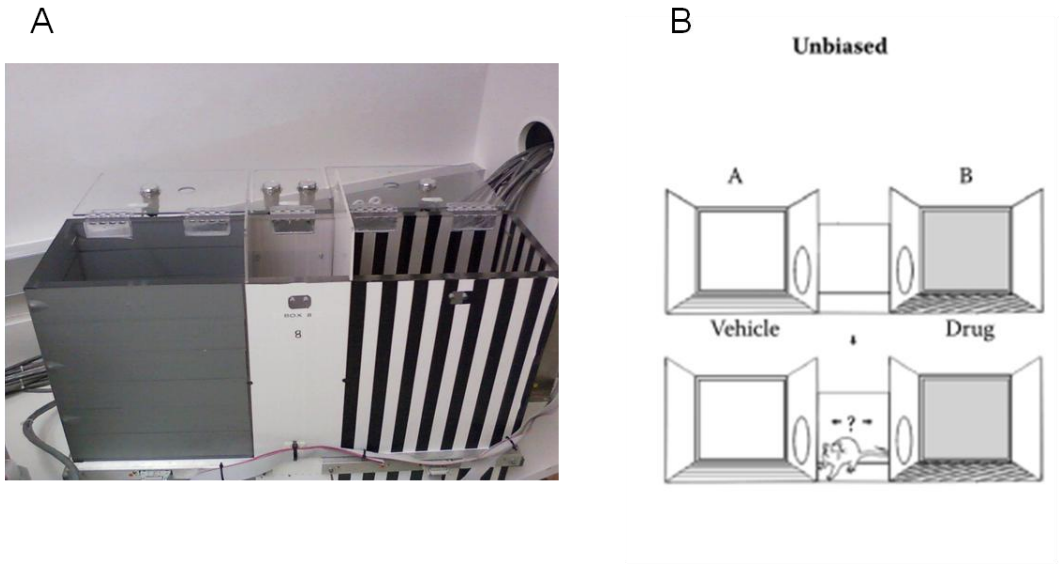

Figure 2.1 CPP Apparatus and protocol design. (A) Picture of three-chamber CPP apparatus used in these studies. Opposite ends of the box have different visual and tactile cues. (B) An unbiased approach, where the drug and vehicle are paired randomly to an environment regardless of baseline behavior, was used. During a pretest session, the animal is allowed to explore all the compartments of the CPP box and a baseline preference/behavior can be determined. Drug and vehicle are assigned to a specific compartment for the conditioning sessions. On the test day, the animal is again placed in the center of the box and allowed to explore all the compartments. If the drug (cocaine) used has positive reinforcement effects, then the animal would spend more time in the drug-paired context than in the vehicle-paired context. Panel B adapted from Prus et al., 2009).

Molecular and biochemical techniques

Several techniques were used to determine changes in gene and protein expression, as well as transcription factor binding to promoter regions, as a result of repeated exposure to cocaine or gene knockdown procedures, such as, Real time reverse transcriptase-PCR, Western Blot or Chromatin Immunoprecipitation (ChIP). Furthermore, viral-mediated gene knockdown was used to manipulate the expression of several circadian genes and study its effects on behavioral and molecular outcomes. Immunohistochemistry (IHC) was used to determine the localization of viral injections in the mouse brain and confirm correct placement in the desired brain region, such as the NAc.

Real time RT-PCR

Real time RT-PCR was used to determine changes in circadian, as well as dopamine receptor, gene expression following 7d of repeated cocaine exposure (15 mg/kg, ip). Mice were euthanized by rapid decapitation and brains were frozen on dry ice and stored at -80°C until used. Brains were sectioned (300µm) and frozen tissue punches were collected from the desired regions, NAc and CP, and stored at -80°C further use. Frozen tissue samples were subjected to RNA isolation using TRIzol reagent (Invitrogen) and following manufacturer's instructions. RNA concentration was determined by using a nano-drop (Thermo Scientific). 1µg of RNA was subjected to DNase treatment (Invitrogen) before cDNA synthesis to avoid any genomic DNA contamination. 1µl of 10X DNase I

Reaction Buffer was added to each sample, followed by 1µl of DNase and left incubating at room temperature for 15min. 1µl of EDTA was added to inactivate the reaction and the samples were incubated at 65°C for 10 min.

cDNA was synthesized using Superscript III (Invitrogen) following manufacturer's instructions. 10µl of RNA was added to a PCR plate, followed by 1µl of Oligo dT primers, 1µl 10mM dNTP Mix, and1µl RNase/DNase-Free H2O. Samples were then incubated at 65°C for 5 min. After incubating samples on ice for at least 1min, Superscript III components were added to the mixture: 4µl 5X First strand buffer, 1µl 0.1 M DTT, 1µl RNaseOUT Recombinant RNase inhibitor, and 1µl of Superscript III RT. Samples were incubated at 50°C for 50min, followed by 15min at 75°C to inactivate the reaction. Samples were diluted to 200µl and stored at -80°C until real time RT-PCR was done. Table 2.1 shows all of the primer sequences used throughout this thesis. 5µl of cDNA was mixed with 1µl of forward and reverse primers (Table 2.1), and 10µl SYBR Green Master Mix. Reactions were run in duplicate in an ABI 7500 Real-Time PCR system (Applied Biosystems).

Chromatin Immunoprecipitation (ChIP)

The ChIP assay is a powerful tool to study protein:DNA interactions *in vivo.* The technique allows to determine whether a gene regulatory protein, in this case transcription factors CLOCK and NPAS2, is bound to a specific DNA sequence, like the promoter region of a certain gene.

Gene	Forward Primer	Reverse Primer
mPer1	5’-CTCTGTGCTGAAGCAAGACCG-3’	5’-TCATCAGAGTGGCCAGGATCTT-3’
mPer2	5’-GAGTGTGTGCAGCGGCTTAG-3’	5’-GTAGGGTGTCATGCGG AAGG-3’
mPer3	5’-GTCCATCTGGAGAATGATAGAGCG-3’	5’-GCTTCAGCACCTCCTCTCGAC-3’
Clock	5’-CAGAACAGTACCCAGAGTGCT-3’	5’-CACCACCTGACCCATAAGCAT-3’
Npas2	5’-GACACTGGAGTCCAGACGCAA-3’	5’-AATGTATACAGGGTGCGCCAAA-3’
DRD1	5’-TGGCATACCTAAGCCACTGGAGAA-3’	5’-ATTCAGGTTGAATGCTGTCCGCTG-3’
DRD2	5’-TCTTCTGGTGGCCACACTGGTTAT-3’	5’-ACAGGTTCAAGATGCTTGCTGTGC-3’
DRD3	5’-AGACACATGGAGAGCTGAAACGCT-3’	5’-TTCAGGGCTGTGGATAACCTGCC-3’
mPer1 promoter	5’-CCTCCTCTAAGGGAAACACCA-3”	5’-GCAAGTGAAGAGGCCAACAC-3’
mPer2 promoter	5’-GCAGCATCTTCATTGAGGAACC-3’	5’-CTCCGCTGTCACATAGTGGAAAACGTGA-3’
mPer3 promoter	5’-CATCTTAGGCTTTCTTGACTTTGAG-3’	5’-CAGAGAGCAAGTATCCACATTTCAT-3’
DRD1 promoter	5’-CCTCAAATTGCCCTCAGGTC-3’	5’-CCCTCGTTTGAAATGCCCCT-3’
DRD2 promoter	5’-TGGCAAGTGATAGAGGCAGG-3’	5’-ACGTCTGAAAATCACAGCGGT-3’
DRD3 promoter	5’-AGGACCTGAGTTCAGATCCCA-3’	5’-TATGACTGTGGGCGAACACA-3’
Gapdh	5’-AACGACCCCTTCATTGAC-3’	5’-TCCACGACATACTCAGCAC-3’
Cyclophilin	5’-CATCTATGGTGAGCGCTTCCCA-3’	5’-GCCTGTGGAATGTGAGGGGTG-3’

Table 2.1 Primer sequences used for real-time RT-PCR

Additionally, it is used to study epigenetic modifications that modulate chromatin remodeling and gene expression. In these studies, ChIP was used to determine the binding of CLOCK and/or NPAS2 to the promoter regions of the *Per* genes and the dopaminergic receptors DRD1, DRD2, and DRD3.

Brain tissue was processed as previously reported (Enwright et al., 2010; Figure 2.2). Animals were euthanized by rapid decapitation and brain tissue, either whole striatum or specific NAc/CP punches, was immediately cross-linked in 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The tissue was washed 5 times in cold PBS containing Complete Protease Inhibitor Cocktail (Roche) and then frozen on dry ice. The chromatin was solubilized and extracted by detergent lysis followed by sonication. First, fixed tissue was homogenized for 10 sec in a cell lysis buffer (10 mM Tris, 10mM NaCl, 0.2% Nonidet P-40). Next, the extracted chromatin was sheared to roughly 500-1,000 bp using the Sonic Dismembrator 550 (Fisher, Hampton, NH). Each sample was sonicated 8 times on ice, 20 sec each, at 25% of maximum power.

Chromatin immunoprecipitation assays were performed according to a protocol outlined in Upstate Biotechnology Inc. ChIP Kit, with some modifications. After the chromatin lysate was extracted and properly fragmented, the optical density of each sample was determined. Equal amounts of chromatin lysate, 60 µg for controls and 100µg for transcription factor IP, were diluted with ChIP dilution buffer (Upstate Biotechnology) to a final volume of 1.1 ml.

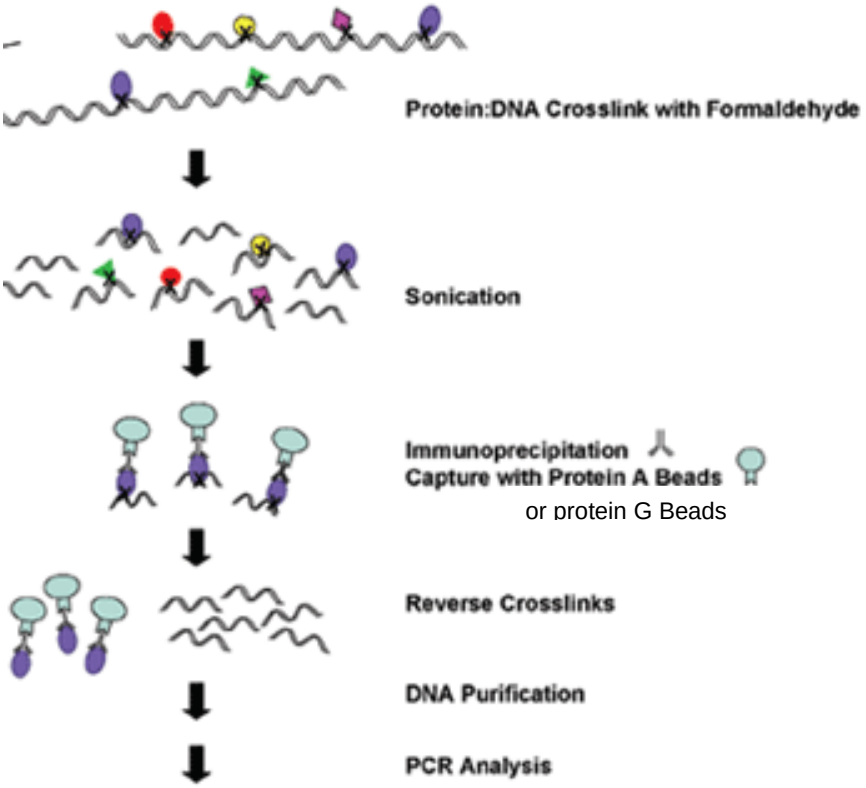

Figure 2.2 Chromatin Immunoprecipitation (ChIP). Brain tissue is fixed with a reversible cross-linking reagent (e.g. 1% formaldehyde) to form protein:DNA complexes. The chromatin is then mechanically sheared (e.g. by sonication) to generate small protein-DNA fragments. These fragments are immunoprecipitated with antibodies specific for the protein of interest and Protein A or G agarose beads. Following immunoprecipitation, the crosslinks are reversed and the DNA is isolated. The isolated DNA can then be analyzed using primers designed to amplify a known regulatory region. Adapted and modified from Affymetrix Inc., 2012.

100 µl of the pre-immunoprecipitated lysate was saved as “input” for later normalization. The chromatin solution was pre-cleared with either salmon sperm DNA/protein A-agarose 50% gel slurry (Pierce, Rockford, IL) or Protein G Agarose/Salmon Sperm DNA (Upstate, Temecula, CA) for 45 min at 4°C. It was then immunoprecipitated overnight at 4°C with an antibody directed against a specific protein either CLOCK H-276 X or NPAS-2 H-20 X (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, samples were immunoprecipitated with non-immune rabbit IgG (Upstate) or Anti-acetyl-Histone H3 (Upstate, Lake Placid, NY). Following immunoprecipitation, the DNA-histone complex was collected with either 40µl salmon sperm DNA/protein A-agarose beads (Pierce, Rockford, IL) or 50µl Protein G Agarose/Salmon Sperm DNA (Upstate, Lake Placid, NY) for 2 hr. The beads were sequentially washed once with low salt, high salt, LiCl, and twice with TE buffers. The DNA-protein complex was then eluted from the beads with 500 µl $NaHCO_3$/SDS elution buffer. Proteins were reverse cross linked from DNA using Proteinase K (Invitrogen) under high-salt conditions at 65°in an overnight incubation. The DNA, associated with a particular transcription factor, was extracted with phenol/chlorophorm/isoamyl alcohol, precipitated with 100% ethanol, washed with 70% ethanol, and finally resuspended in 0.1X TE Buffer diluted in PCR-grade water. Levels of specific transcription factor binding or histone modifications at each gene promoter of interest were determined by measuring the amount of associated DNA by real-time PCR (ABI 7500 , Foster City, CA). Input or total DNA (non-

immunoprecipitated) and immunoprecipitated DNA were amplified in duplicate in the presence of SYBR Green (Applied Biosystems). Relative quantification of template DNA was performed using the ΔΔCt method. Significance was determined by ANOVA.

Gene knockdown via RNA interference (RNAi): Construction of shRNAs directed toward specific circadian genes

A small hairpin RNA (shRNA) was constructed against *mPer1* and *mPer2* mRNA by selecting a conserved 24 base sequence (5'- ATCCCTCCTGACAAGA GGA TCTTC-3') in the coding region. For the *Npas2* gene, a shRNA was constructed by selecting a 24 base sequence (5'-GAACACTGGATTCTTCCTGT TAAC -3') in the 3'-UTR. The Clock shRNA was previously published by members of the laboratory (Mukherjee et al., 2010). For the scrambled shRNA, a random sequence of 24 bases (5'-CGGAATTTAGTTACG GGGATCCAC-3') that had no sequence similarities with any known genes/mRNA was used. An antisense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotides (CTTCCTGTCA) was added at the 5'end of the above sequences. The miR23 loop facilitates the transfer of the hairpin RNA out of the nucleus. These shRNAs were designed as synthetic duplexes with overhang ends identical to those created by Sap I and Xba I restriction enzyme digestion. The annealed oligonucleotides were cloned into the adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (EGFP) (Stratagene, La

Jolla, CA). Plasmids were sent to the University of North Carolina Viral Vector Core for production (Chapel Hill, NC).

Immunohistochemistry (IHC)

This technique was used to visualize viral expression in the NAc. AAV vectors containing shRNA sequences (see above), also encode for EGFP, allowing for easy localization of the viral infusion. Mice were anesthetized with 50 mg/kg Nembutal in saline, and transcardially perfused with 4% paraformaldehyde in 1X PBS (1 mM KH2PO4, 10 M Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The brains were allowed to post-fix in a 4% paraformaldehyde for 24 hours and then placed in 1X PBS-30% glycerol sucrose protection for an additional 24 hours before being stored in 1X PBS-0.05% sodium azide. 30 µm brain sections were obtained with a microtome (Leica, Wetzlar, Germany) and immunohistochemical staining against GFP (AbCam, Cambridge, MA) was carried out using standard procedures. Secondary antibodies (anti-rabbit conjugated with Alexa 488) were purchased from Molecular Probes (Carlsbad, CA). Brain sections were mounted using Vectashield (Vector Labs, Burlingame, CA) with DAPI counterstaining and observed with an epifluorescence microscope with a 10x objective. Animals were excluded from our study if their infection spread was not localized to the NAc , with spillover to adjacent areas or throughout the injection tract; or if there was a significant disproportionate amount of infection between both hemispheres. Exclusion by these criteria accounted for approximately 10-15% of animals.

Western Blot

Western Blot is a widely used technique utilized to study changes in protein expression levels. Here, it was used to determine changes in PER protein levels following acute or repeated cocaine exposure. Western blot assays were run as published previously (Hope *et al.* 1994). After chronic cocaine treatment, tissue punches were taken and frozen at -80°C until used. Tissue was sonicated on ice in a modified detergent based buffer containing both phosphatase and protease inhibitors (Roche, Sigma). After sonication, samples were denatured in boiling water and centrifuged at 15,000xg for 15 minutes, supernatant was subsequently collected and processed; protein concentration amounts were then quantified using a Bradford assay (Bio-Rad). Samples were run on a 10% acrylamide/bisacrylamide gel, transferred to a PVDF membrane, blocked in 5% milk and incubated with primary antibodies (PER 1, 2, and 3 Chemicon, Temecula, CA,). Blots were subsequently visualized using a chemiluminescence system (Pierce). All samples were normalized to GAPDH (Fitzgerald, Concord, MA). Standard curves were run to ensure that we are in the linear range of the assay. Densitometry was conducted using NIH image software and samples are expressed as a percent change from control.

CHAPTER THREE

DIFFERENTIAL REGULATION OF THE *PERIOD* GENES BY COCAINE IN STRIATAL REGIONS

Introduction

Drug addiction is associated with major disruptions in circadian rhythms. For example, drug addicts are commonly reported to have disruptions in their sleep/wake cycle, activity cycles, eating habits, as well as, blood pressure, hormone secretion and body temperature rhythms (Wasielewsky et al., 2001; Jones et al., 2003). Genes that are important in regulating drug-induced behaviors are often induced or repressed throughout striatal regions by drugs of abuse (Nestler, 2005). Indeed, various studies have found changes in the expression of circadian genes in striatal regions in response to psychostimulants. Interestingly, these changes are often specific to a given region or treatment. For example, *rPer1* is induced in the rat CP following acute cocaine, while *rPer2* is induced following a chronic "binge" pattern of cocaine (Yuferov et al., 2003). Another study showed that acute methamphetamine treatment leads to a rapid induction of *mPer1*, but not *mPer2* or *mPer3* expression in the mouse CP (Nikaido et al., 2001). This suggests that the induction of circadian genes in these regions is specific, or that *mPer1* responds to cocaine as an immediate early gene. Indeed, rapid induction of *mPer1* is seen in the SCN in response to light (Crosio et al., 2000). Furthermore, a study by Lynch *et al* found that seven

days of cocaine self-administration lead to a significant upregulation of a number of circadian and circadian-associated genes in the dorsal striatum, including *Clock, Bmal1, Per2,* and *Cryptochrome1 (Cry1)*, among others (Lynch et al., 2008). These studies suggest that psychostimulants have selective effects on the expression of these genes in striatal regions. However, it is important to note that many, if not all, of these previous studies have looked at expression of circadian genes at only a specific timepoint, when in fact the *Per* genes are known to have rhythmic and different temporal profiles of expression in limbic regions (Lamont et al., 2005). Thus, it is also important to look at the expression of these genes throughout a 24 hr period in order to distinguish changes in rhythm amplitude or phase from specific drug-induced timepoint-dependent effects.

Even though the master pacemaker is located in the SCN, circadian genes and proteins are widely expressed throughout the brain, thereby forming SCN-independent pacemakers that entrain to other non-photic stimuli such as food (Iijima et al., 2002; Stephan, 1984). Drugs of abuse can also serve as powerful Zeitgebers for some of these clocks outside of the SCN. Several studies have shown that drugs of abuse, like cocaine, methamphetamine, nicotine and alcohol, can entrain locomotor activity rhythms (Kosobud et al., 2007). In these studies, animals started to develop what was termed circadian activity episodes or an anticipatory increase in locomotor activity 22-23 hrs after the last injection or, in other words, 1-2 hrs before the next injection time (Kosobud et al., 2007; Gillman et al., 2008; Gillman et al., 2009). This behavioral entrainment effect has only been observed after administration of rewarding

substances. While animals have also been found to entrain to fentanyl, a synthetic opioid, they fail to develop anticipatory locomotor activity to repeated doses of haloperidol, a substance that is not rewarding (Gillman et al., 2009). Interestingly, studies have suggested that this is not just an effect of regular interval learning, since animals injected in a 31 or 33-hr schedule will still exhibit a circadian ensuing episode of activity 24hrs after the injection and not immediately prior to the administration time (White et al., 2000; Pecoraro et al., 2000; Gillman et al., 2009) . This suggests that entrainment is related specifically to circadian factors rather than a conditioned Pavlovian interaction with circadian rhythms. It is important to note that these circadian activity episodes persist for several days after drug has been withheld, further demonstrating the ability of drugs of abuse to entrain behavioral rhythms. Furthermore, in rodents with a lesioned SCN, methamphetamine in the drinking water restores activity rhythms in a robust manner, and animals can be entrained to daily methamphetamine injections (Iijima et al., 2002; Masubuchi et al., 2000). In addition, methamphetamine treatment shifts the expression of the *Per* genes in striatal regions in a manner that matches the shifts in activity rhythms and is independent from the SCN rhythms (Iijima et al., 2002).

Both NPAS2 and CLOCK can heterodimerize with BMAL1 to activate transcription of the *Per* and *Cry* genes among other clock-controlled genes (Reick et al., 2001). However, while CLOCK is widely expressed throughout the brain, including high levels in the SCN, the expression of NPAS2 is restricted to the forebrain, with reduced expression in the SCN under normal conditions, but

with high levels in striatal regions (King et al., 1997; Zhou et al., 1997; Garcia et al., 2000). NPAS2 has little to no expression in the VTA, but since it has high levels of expression in both CP and NAc, which receive input from midbrain dopaminergic regions, NPAS2 may also be involved in regulating the response to drugs of abuse, as well as, the regulation of *Period* expression in the striatum. This study investigated the differential regulation of the *Period* genes by cocaine, the role of Npas2 in this regulation, and the effect of repeated cocaine injections on molecular rhythms of circadian genes in striatal regions.

Materials and Methods

Animals

C57BL/6 mice (The Jackson Laboratory) were group housed in a 12/12 light/dark (LD) cycle (lights on at 7am, lights off at 7pm) with food and water *ad libitum*. For the 24-hr time series studies, mice were group housed under the same LD schedule in temperature-controlled and sound-proof cabinets. Male 8-week old mice were used in all studies. All animal use was approved by the UTSW Institutional Animal Care and Use Committee.

Drug

Cocaine Hydrochloride (HCl) was generously provided by the National Institute on Drug Abuse. Animals were injected with a 15mg/kg cocaine or saline i.p. in all studies.

Real time RT-PCR

RNA was isolated from mechanically homogenized tissue using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. This was followed by a 15 min treatment with DNAse I (Invitrogen) according to manufacturer's protocols to digest any remaining genomic DNA. One microgram of total RNA was used to synthesize cDNA using Superscript III Reverse Transcriptase (Invitrogen) per the manufacturer's instructions. cDNA or chromatin samples were mixed with SYBR Green master mix (Applied Biosystems, ABI) and specific primers for genes or promoter regions of interest (for a list of primer see supplemental materials). Prior to the experiment primer sets were tested thoroughly to determine reaction efficiency, specificity, and the absence of primer-dimers. Reactions were run on an ABI Prism 7700 real-time PCR machine. Fold changes and relative gene expression were calculated using the comparative Ct method and normalized to the corresponding *Gapdh* or *Cyclophilin* mRNA levels. The Ct values used for these calculations are the mean of at least four biological replicates of the same reaction; each PCR reaction was done in duplicate and used 5µl of cDNA.

Western Blot

Western Blot assays were run as previously published (Hope et al., 1994). After chronic cocaine treatment, tissue punches were taken and frozen at -80°C until used. Brain tissue was sonicated on ice in a modified detergent based buffer containing both phosphatase and protease inhibitors (Roche, Sigma). After

sonication, samples were denatured in boiling water and centrifuged at 15,000xg for 15 min, supernatant was subsequently collected and processed; protein concentration amounts were then quantified using a Bradford assay (Bio-Rad). Samples were run on a 10% acrylamide/bisacrylamide gel, transferred to a PVDF membrane, blocked in 5% milk and incubated with primary antibodies (PER 1,2,3 Chemicon). Blots were subsequently visualized using a chemiluminescence system (Pierce). All samples were normalized to GAPDH (Fitzgerald, Concord, MA). Standard curves were run to ensure that we are in the linear range of the assay. Densitometry was conducted using NIH image software.

Chromatin Immunoprecipitation (ChIP)

Brain tissue was processed as previously reported (Enwright et al., 2010). Brain punches were taken in NAc and CP regions from 8 mice and pooled, and immediately cross-linked in 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125M. The tissue was washed 5 times in cold PBS containing Complete Protease Inhibitor Cocktail (Roche) and then frozen on dry ice. The chromatin was solubilized and extracted by detergent lysis, followed by sonication. First, fixed tissue was homogenized twice, for 10 sec, in a cell lysis buffer (10mM Tris, 10mM NaCl, 0.2% Nonidet P-40). Next, the extracted chromatin was sheared to roughly 500-1,000 bp using the Sonic Dismembrator 550 (Fisher, Hampton, NH). Each sample was sonicated 8 times on ice, 20 sec each, at 25% of maximum power. After the chromatin lysate was extracted and properly fragmented, the

optical density of each sample was determined. Equal amounts of chromatin lysate, 60 µg, were diluted with ChIP dilution buffer (Upstate) to a final volume of 1.1 ml. 100 µl of the pre-immunoprecipitated lysate was saved as “input” for later normalization. The chromatin solution was pre-cleared with either salmon sperm DNA/protein A-agarose gel slurry (Thermo Scientific) or Protein G Agarose/Salmon Sperm DNA (Thermo Scientific) for 45 min at 4°C. It was then immunoprecipitated overnight at 4°C with an antibody directed against a specific protein either CLOCK H-276 X or NPAS-2 H-20 X (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, samples were immunoprecipitated with non-immune rabbit IgG (Upstate) or Anti-acetyl-Histone H3 (Upstate). Following immunoprecipitation, the DNA-protein complex was collected with either 40µl salmon sperm DNA/protein A-agarose beads or 50µl Protein G Agarose/Salmon Sperm DNA for 2 hr. The beads were sequentially washed once with low salt, high salt, LiCl, and twice with TE buffers. The DNA-protein complex was then eluted from the beads with 500 µl $NaHCO_3$/SDS elution buffer. Proteins were reverse-cross linked from DNA using Proteinase K (Invitrogen) under high-salt conditions at 65°C for at least 4 hr. The DNA, associated with a particular transcription factor, was extracted with phenol/chlorophorm/isoamyl alcohol, precipitated with 100% ethanol, washed with 70% ethanol, and finally resuspended in 0.1X TE Buffer diluted in PCR-grade water. Levels of specific transcription factor binding or histone modifications at each gene promoter of interest were determined by measuring the amount of associated DNA by real-time PCR (Applied Biosystems (ABI) Prism 7700, Foster City, CA). Input or total

DNA (nonimmunoprecipitated) and immunoprecipitated DNA were amplified in duplicate in the presence of SYBR Green (ABI). Relative quantification of template DNA was performed using the comparative Ct method.

Data Analysis

Single timepoint studies, immunoblots, and ChIP assays were analyzed by Student's t-test using Bonferroni adjustments for multiple comparisons (Figures 3.1 – 3.4). For the time series experiments, one-way ANOVAs followed by Tukey's Multiple Comparison Test were used to establish diurnal rhythmicity for each gene and each condition, as previously published (Maywood et al., 2010). Additionally, two-way ANOVAs followed by post-hoc t-tests were used to assess the overall effect of treatment and time.

Results

Npas2 expression is induced by chronic cocaine in striatal regions while *Clock* and *Bmal1* expression is unchanged.

To better understand the molecular actions of CLOCK and NPAS2 in striatal regions, we administered acute cocaine (15mg/kg, i.p.), chronic cocaine (15 mg/kg, i.p., once/day for 7 days), or saline, and examined the expression levels of *Clock*, *Npas2*, and the binding partner for both genes, *Bmal1,* 24 hours after treatment.

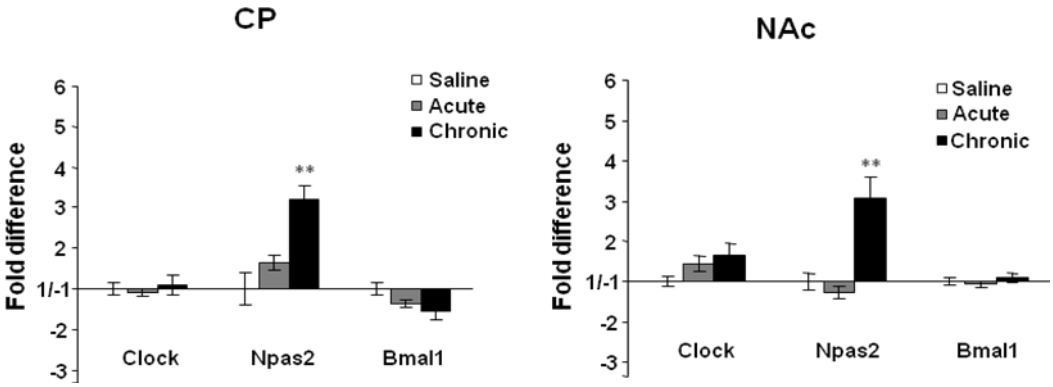

Figure 3.1 *Clock*, *Npas2* and *Bmal1* expression after cocaine treatment. Real-time PCR analysis of *Clock, Npas2,* and *Bmal1* expression in the CP and NAc following saline, acute (15 mg/kg, 1 day), or chronic cocaine treatment (15 mg/kg, 7 days) in wild type mice. ***P*<0.01 by t-test, n=6.

This chronic treatment paradigm has been used in previous studies to elicit cocaine sensitization and mediate long term changes in cocaine responsive genes and proteins (Nestler 2004). Interestingly, we found that *Npas2* mRNA expression was upregulated in both the NAc and CP by chronic but not acute cocaine exposure (Figure 3.1). Expression levels of *Clock* and *Bmal1* were not significantly regulated by cocaine in these regions following these treatment paradigms (Figure 3.1). These results suggest that chronic cocaine treatment selectively affects the expression of *Npas2* in striatal regions.

The *Period* genes are differentially induced by cocaine in striatal regions

Both NPAS2 and CLOCK regulate the expression of the *Per* genes (*Per1, and 2*) and *Cryptochrome* genes (*Cry1* and *2*) (Gekakis *et al.* 1998, Reick et al. 2001). These proteins serve as inhibitors of NPAS2/BMAL1 or CLOCK/BMAL1 function in transcriptional feedback loops throughout the brain (Ko & Takahashi 2006). There have been reports implicating *Per1* and *Per2* in the behavioral responses to cocaine, methamphetamine, morphine, and alcohol (Abarca et al. 2002, Spanagel et al. 2005, Masubuchi *et al.* 2000, Manev & Uz 2006, Yuferov *et al.* 2005, Nikaido et al. 2001, Liu *et al.* 2005). However, it appears that they may serve differential functions in response to these drugs. Therefore, we wanted to determine if these genes are differentially regulated by acute or chronic cocaine in the NAc or CP. We again administered acute cocaine (15mg/kg, i.p.), chronic cocaine (15 mg/kg, i.p., once/day for 7 days), or saline and examined the

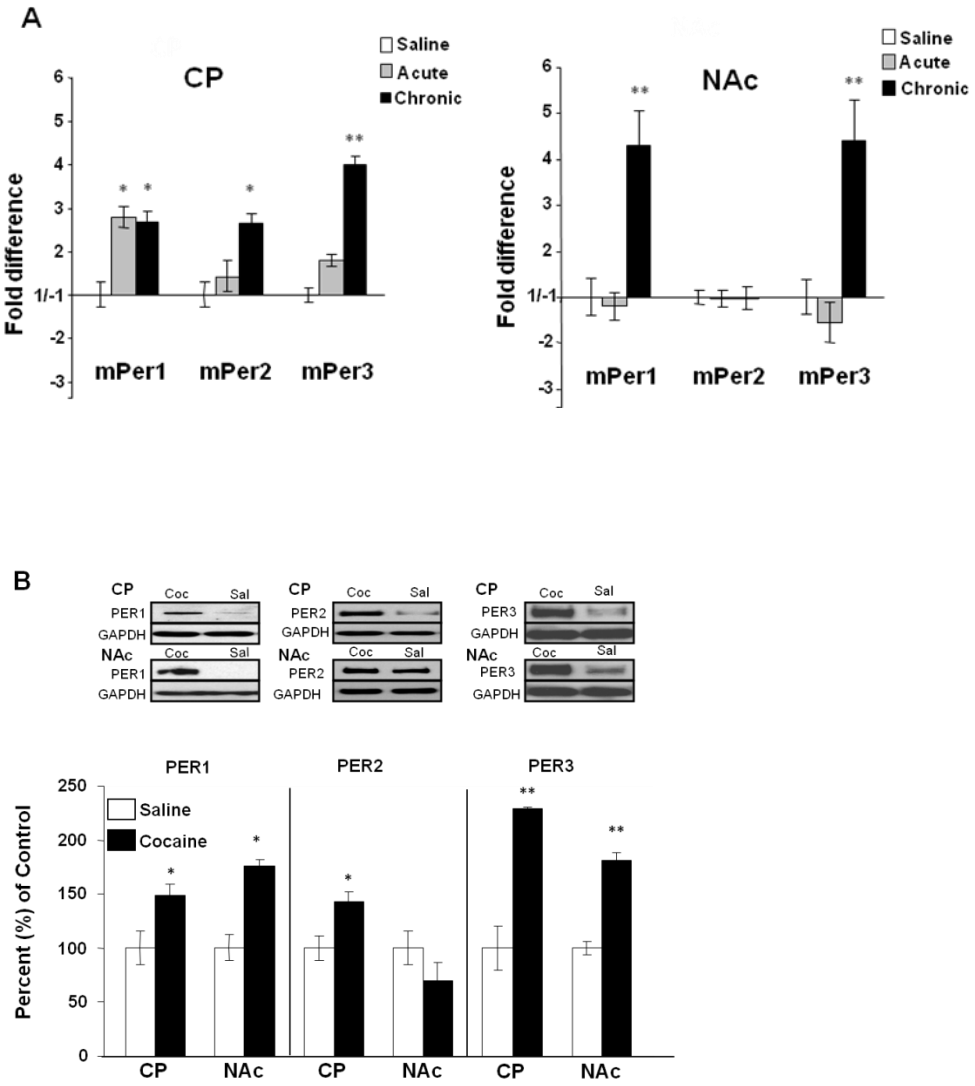

Figure 3.2 *Per* gene and protein expression after cocaine treatment. (A) Real-time PCR analysis of *mPer1, mPer2* and *mPer3* expression in the CP and NAc following saline, acute (15 mg/kg, 1 day), or chronic cocaine treatment (15 mg/kg, 7 days) in wild type mice. **P*<0.05, ***P*<0.01 by t-test. n=6. (B) Cocaine (15mg/kg) or saline was given chronically (7 days) i.p. Protein levels were measured 24 hrs later using western blot analysis in the CP and NAc. GAPDH was measured as a loading control. n=5-8. Representative blots are shown and the percent change in cocaine vs saline is shown.

expression levels of the *mPer1,m Per2 mPer3, Cry1,* and *Cry2* genes, 24 hours after treatment. Similar to previous studies that measured gene expression changes in the CP with psychostimulant exposure (Yuferov et al. 2003, Nikaido et al. 2001), we found that only *mPer1* in the CP is regulated by acute cocaine, however, all three *Per* genes were upregulated following chronic cocaine (Figure 3.2a). Interestingly, *mPer1* and *mPer3* were also upregulated by chronic cocaine in the NAc while *mPer2* was not regulated in this region at this timepoint (Figure 3.2b). We saw no changes in the expression of the *mCry* genes by these treatments in either brain region (data not shown), suggesting that the paradigms employed selectively affect the *Per* genes.

These regional changes in mRNA expression following chronic cocaine exposure are also seen at the protein level as assessed by western blot analysis, indicating that they likely result in altered PER function (Figure 3.2c and 3.2d). Again, we saw no difference in mPER2 protein levels in the NAc at this timepoint. This regional difference in expression is interesting since the NAc is thought to be involved in drug reward and overall hedonic state while the CP is more involved in the locomotor response to drugs, as well as the habitual and compulsive behaviors associated with addiction and other psychiatric disorders (Everitt & Robbins 2005). These results also suggest that the regulation of these genes may occur through different second messenger pathways and may modulate different behavioral responses associated with mood and addiction.

A mutation in NPAS2 affects the induction of the *Period* genes after cocaine

To determine if NPAS2 is involved in the induction of these genes following chronic cocaine administration, we gave chronic cocaine (15mg/kg, i.p. 7 days) or saline to the *Npas2* mutant mice and looked for changes in *Per* gene expression 24 hours after the last treatment. All inductions of the *Per* genes in the CP after chronic cocaine fail to occur if *Npas2* is disrupted (Figure 3.3). However, while the induction of *mPer3* is also diminished in the NAc, the induction of *mPer1* in this region is unchanged (Figure 3.3). *mPer2* levels in the NAc were unaffected since they are not induced by cocaine in wild type mice. This suggests that NPAS2 is necessary for the induction of all three *Per* genes in the CP following cocaine treatment and *mPer3* in the NAc, however, it is not necessary for the induction of *mPer1* in the NAc. We did not see a significant reduction in baseline *Per* gene expression levels in the *Npas2* mutant mice versus wild type controls at this timepoint. The expression levels of these genes in striatum are normally low at the time of day that these experiments were performed (between ZT 3-6) (Iijima et al., 2002; Masubuchi et al., 2000; Reick et al., 2001), therefore a further reduction in expression is difficult to assess, and has not been seen in previous studies (Reick et al., 2001). However, the peak levels of *mPer2* expression (occurring during the subjective night) are dampened in these mice under baseline conditions, selectively in regions of the brain that express NPAS2 (Reick et al., 2001).

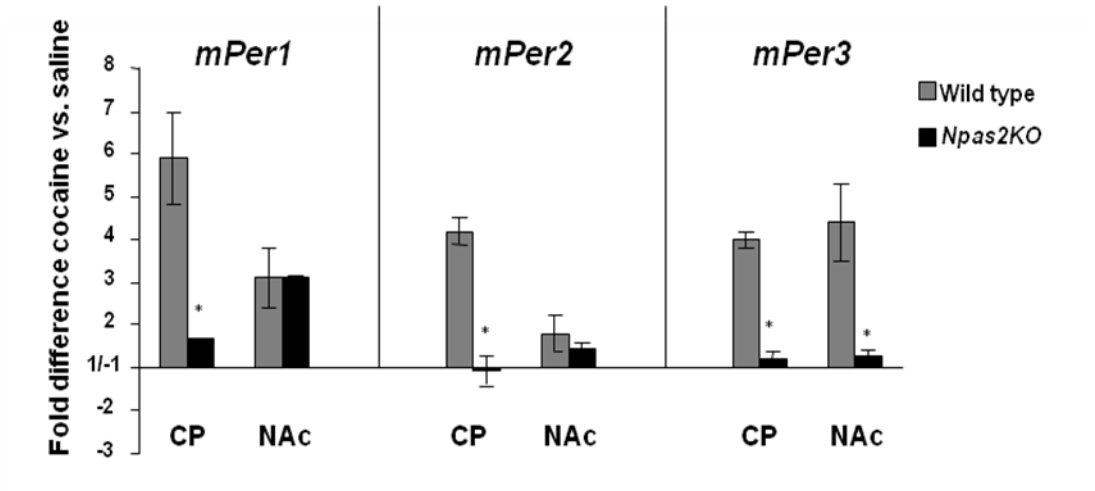

Figure 3.3 Effect of the *Npas2* mutation on *Per* gene induction following cocaine. Real-time PCR analysis of *mPer1, mPer2, and mPer3* expression in the CP and NAc following chronic treatment (15 mg/kg, 7 days) with saline or cocaine in wild type and *Npas2* mutant mice. Shown are the fold changes in cocaine treated animals relative to saline treated animals. **P*<0.01 by t-test. n=5.

Cocaine treatment is associated with an increase in NPAS2 binding, but not CLOCK binding to the *Period* gene promoters in the CP and NAc.

To better understand the molecular actions of repeated cocaine administration on the regulation of *Per* gene expression, we employed chromatin immunoprecipitation assays (ChIP) to determine if NPAS2 and CLOCK are binding to the promoter regions of the *Per* genes in striatal regions, and if cocaine treatment affects this binding. Wild type mice were administered either chronic cocaine (15 mg/kg, i.p., once/day, 7 days) or saline treatment. ChIP assays using CP and NAc tissue were then performed with antibodies directed against either NPAS2 or CLOCK. Chronic cocaine treatment lead to a dramatic and selective increase in the binding of NPAS2 and not CLOCK to all three *Per* gene promoters in the CP (figure 3.4A and 3.4B). There was also a significant increase in binding of NPAS2 to the *mPer1* and *mPer3* promoters in the NAc with no increase in CLOCK binding (figure 3.4A and 3.4B). Interestingly, there was no increase in the binding of NPAS2 to the *mPer2* promoter in the NAc following cocaine treatment, which correlates with the lack of induction of this gene or protein by this treatment. These results suggest that chronic cocaine treatment leads to a selective induction of NPAS2 binding at certain *Per* gene promoters leading to an increase in their expression.

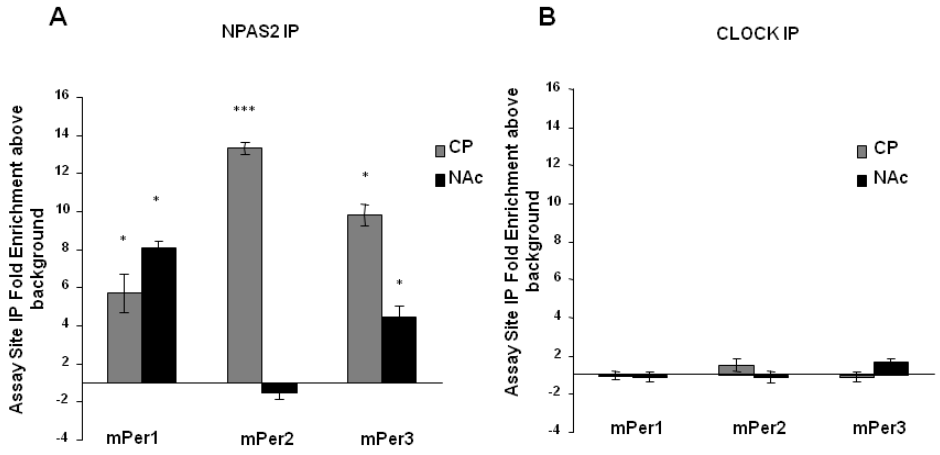

Figure 3.4 Binding of NPAS2 and CLOCK to the *Per* promoters in striatal regions. ChIP assays were performed with antibodies specific to CLOCK, NPAS2, and acetylated histone H3. IgG was used as a negative control. This was followed by real-time PCR analysis using primers specific to the *mPer1*, *mPer2*, or *mPer3* promoters.(A) ChIP assays were performed in NAc and CP tissue using an antibody for NPAS2 in animals treated chronically with cocaine (15mg/kg, 7 days) or saline. Shown are the fold changes in cocaine treated animals versus saline treated animals. (B) ChIP assays were performed in NAc and CP tissue using an antibody for CLOCK in animals treated chronically with cocaine (15mg/kg, 7 days) or saline. Shown are the fold changes in cocaine treated animals versus saline treated animals (Fold ±1). *P<0.05, ***P<0.001 by t-test (cocaine vs. saline), n=8.

Chronic cocaine treatment alters molecular rhythms in striatal regions

Since all of the gene expression studies were performed at a single timepoint, it was of interest to know whether chronic daytime cocaine treatment leads to an increase in *Npas2* and *Per* gene expression across the light/dark cycle while maintaining a normal rhythm, or if it is part of an alteration in their molecular rhythms. Mice were treated with cocaine (15 mg/kg) or saline i.p. for 7 d at ZT 6 (1pm). Animals were sacrificed starting 26 hrs after the last injection (ZT8, 3pm) and subsequently every 4 hrs until ZT4 (11am). This last timepoint is exactly 2 hrs prior to drug administration time and thus, might provide some insight into possible anticipatory effects. *Clock* exhibits diurnal rhythmicity in both striatal regions under saline conditions ($F_{(5,41)}$=5.389, p=0.0007 NAc; $F_{(5,36)}$=4.216, p=0.0042 CP), which are not affected following chronic cocaine treatment ($F_{(5,40)}$=6.120, p=0.0003 NAc; $F_{(5,35)}$=2.937, p=0.0257 CP) (Figure 3.5A). A two-way ANOVA revealed a lack of effect of treatment and interaction, but a highly significant effect of time in both regions ($F_{(5,81)}$=9.82, p<0.0001 NAc; $F_{(5,69)}$=5.83, p=0.0002 CP).Even though no interaction effect was observed, a significant difference was observed at ZT 20 in the NAc (*p<0.05) and a trend towards upregulation was observed at ZT 24 in the CP. *Npas2* diurnal rhythmicity in the NAc under saline conditions is abolished following chronic cocaine treatment ($F_{(5,40)}$=6.720, p= 0.0001 saline; $F_{(5,43)}$=1.120, p=0.3641 cocaine).

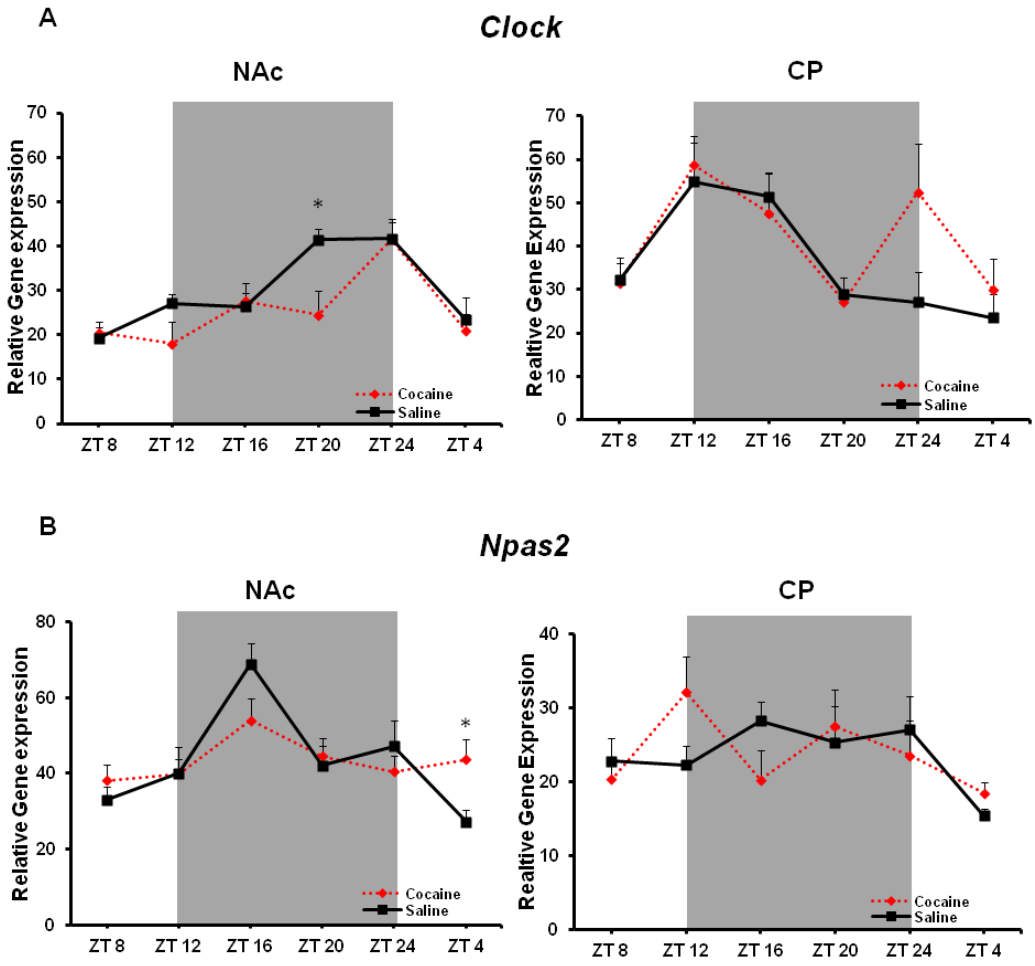

Figure 3.5 Chronic cocaine alters *Npas2* rhythmic expression only in the NAc, whereas *Clock* is unchanged. (A) *Clock* gene expression (mean ± SEM, n= 6-9) in the NAc and CP is unaltered by chronic cocaine (($F_{(5,41)=}$5.389, p=0.0007 NAc and $F_{(5,36)=}$4.216, p=0.0042 CP in saline; $F_{(5,40)}$=6.120, p=0.0003 NAc and $F_{(5,35)}$=2.937, p=0.0257 CP in cocaine). (B) *Npas2* rhythmicity in the NAc is abolished following chronic cocaine ($F_{(5,40)}$=6.720, p= 0.0001 saline; $F_{(5,43)}$=1.120, p=0.3641 cocaine) and a significant upregulation was observed at ZT 4 (*p<0.05). Dark background indicates lights-off.

A two-way ANOVA revealed only a significant effect of time ($F_{(5,82)}$=7.12, p<0.0001 NAc), but no effect of treatment or interaction. There was no significant change over time of *Npas2* expression in the CP under both treatment conditions. A significant upregulation was observed at ZT 4 in the NAc, but not the CP (*p<0.05; Figure 3.5B). Interestingly, no difference was observed at ZT8, just a few hours after a timepoint similar to that in Figure 3.1, suggesting that this effect dissipates by ~26hrs post-injection, but returns a few hours prior drug administration.

mPer1 exhibits diurnal rhythmicity in both regions under control conditions ($F_{(5,27)}$=3.575, p=0.0131 NAc; $F_{(5,36)}$=3.826, p=0.007 CP; Figure 3.6A).Following chronic cocaine, this rhythmicity is lost in the CP, but maintained in the NAc ($F_{(5,33)}$=4.862, p=0.0019). A two-way ANOVA revealed an extremely significant effect of treatment ($F_{(1,60)}$=12.18, p=0.0009) and time ($F_{(5,60)}$=6.52, p<0.0001) in the NAc, as well as a significant interaction ($F_{(5,67)}$=2.48, p=0.0401) and time effect ($F_{(5,67)}$=2.92, p=0.0191) in the CP. Again, an upregulation of *mPer1* was observed following cocaine in both regions at ZT 4 (*p<0.05). *mPer2* rhythmicity is unaffected by cocaine in the NAc ($F_{(5,34)}$=3.839, p=0.0073 saline; $F_{(5,35)}$=3.589, p=0.01 cocaine; Figure 3.6B). In the CP, *mPer2* rhythmicity is disrupted following cocaine treatment ($F_{(5,35)}$=8.273, p<0.0001 saline; $F_{(5,38)}$=2.005, p=0.1 cocaine). In the NAc, a two-way ANOVA revealed a significant interaction effect ($F_{(5,69)}$=2.47, p=0.00404) and a highly significant time effect ($F_{(5,69)}$=5.08, p=0.0005); as well as a highly significant effect of time ($F_{(5,72)}$=9.13, p<0.0001) in the CP.

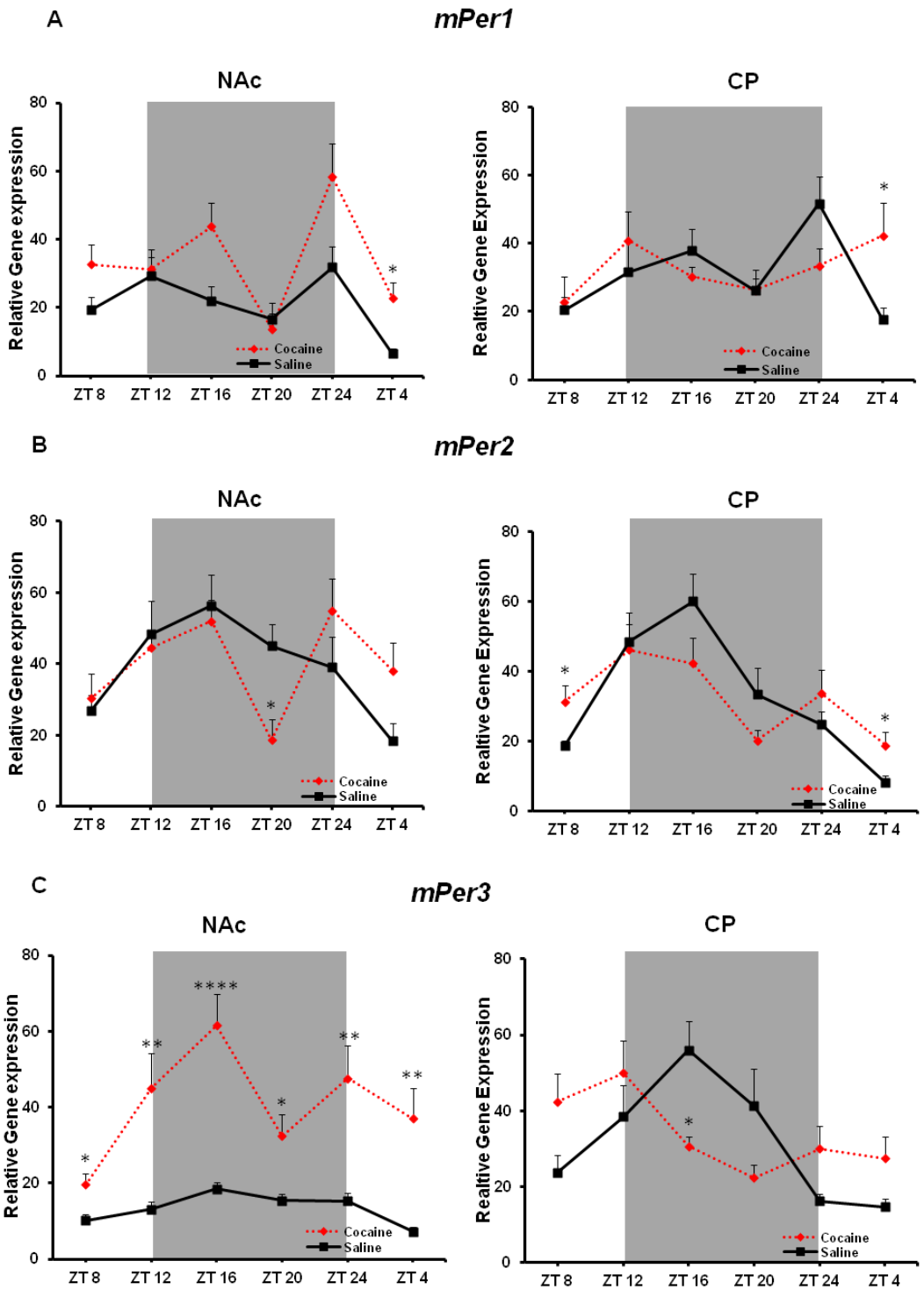
A
mPer1
NAc
CP
Relative Gene expression
Realtive Gene Expression
Cocaine
Saline
ZT 8
ZT 12
ZT 16
ZT 20
ZT 24
ZT 4
B
mPer2
C
mPer3

Figure 3.6 Chronic cocaine alters diurnal expression of *Per* genes in the NAc and CP. (A) Cocaine alters *mPer1* expression (mean ± SEM, n= 6-9) in the NAc and CP. Rhythmicity of *mPer1* is maintained in the NAc but abolished in the CP following cocaine treatment ($F_{(5,27)}$=3.575, p=0.0131 NAc saline; $F_{(5,36)}$=3.826, p=0.007 CP saline; $F_{(5,33)}$=4.862, p=0.0019 NAc cocaine). A significant upregulation at ZT4 was observed in both regions (*p<0.05). (B) *mPer2* rhythmicity is unaltered in the NAc but disrupted in the CP following cocaine ($F_{(5,35)}$=8.273, p<0.0001 saline CP; $F_{(5,38)}$=2.005, p=0.1 cocaine CP). A significant upregulation at ZT 4was observed only in the CP (*p<0.05). (C) While rhythmicity is maintained, *mPer3* is highly upregulated in the NAc ($F_{(5,40)}$=5.176, p=0.0009 saline; $F_{(5,36)}$=4.135, p= 0.0045 cocaine; *p<0.05, **p<0.01, ****p<0.0001). Cocaine disrupts rhythmicity of *mPer3* in the CP ($F_{(5,33)}$=5.077, p=0.0015 saline; $F_{(5,35)}$=2.343, p=0.0617 cocaine).

An upregulation at ZT4, similar to that previously observed, and ZT8 were observed in the CP and not the NAc following cocaine (*p<0.05). *mPer3* displays a diurnal rhythmicity in both regions under saline conditions ($F_{(5,40)}$=5.176, p=0.0009 NAc; $F_{(5,33)}$=5.077, p=0.0015 CP; Figure 3.6C). After cocaine treatment, rhythmicity is maintained in the NAc ($F_{(5,36)}$=4.135, p= 0.0045) but not in the CP ($F_{(5,35)}$=2.343, p=0.0617). A two-way ANOVA revealed in the NAc a significant interaction effect ($F_{(5,76)}$=3.16, p=0.0121), an highly significant treatment effect ($F_{(1,76)}$=93.59, p<0.0001), and a highly significant effect of time ($F_{(5,76)}$=6.11, p<0.0001). No such effects were detected in the CP; however, there seemed to be a trend towards a shift in the expression of *mPer3*, peaking during the day and not the dark phase. All timepoints in the NAc showed a significant upregulation by cocaine when compared to saline (*p<0.05, **p<0.01, ****p<0.0001). In the CP, a significant timepoint difference was observed only at ZT 20 (*p<0.05).

Discussion

This study found that specific circadian genes/proteins are altered by cocaine treatment in striatal regions. Some of these alterations are not just timepoint-specific effects but rather reflect a change in the overall rhythmic expression of these genes. Both NPAS2 and CLOCK proteins are expressed in the NAc and CP, however, only *Npas2* expression is induced by cocaine in these regions. Furthermore, both NPAS2 and CLOCK bind to the *Per* gene promoters

in striatal regions, though chronic cocaine treatment only affects the binding of NPAS2 at these promoters. Interestingly, the Per genes were differentially regulated by acute and chronic cocaine in both NAc and CP. Similar to previous studies, *mPer1* is induced by acute cocaine while *mPer2* is induced by chronic cocaine (Yuferov et al. 2003, Nikaido et al. 2001). Thus, it appears that *mPer1* functions much like an immediate early gene in response to cocaine while *mPer2* and *mPer3* may be more involved in the chronic effects of cocaine use. *mPer1* also displays the kinetics of an immediate-early gene in response to light in the SCN while *mPer2* responds more slowly (Shearman *et al.* 1999). Therefore, this may be a general property of these proteins in response to many types of stimuli, and this could underlie some of the differences seen in *mPer1* and *mPer2* function. The function of *mPer3* in the circadian system in the SCN appears to be minimal and outside of the central clock (Bae et al., 2001; Pendergast,et al., 2012). Therefore the induction of *mPer3* by cocaine may have completely different consequences than the induction of the other *Per* genes. Interestingly, Pieper *et al* identified about twice as many genetic variations in *hPer3* than *hPer1* in human subjects with psychiatric disorders, suggesting that *Per3* may be more involved in mental illness than *Per1* (Pieper et al, 2006).

The observed cocaine-induced regulation of the *Per* genes was found to be mediated through Npas2, since the response was abolished in Npas2 mutant mice. Another interesting finding is that the increase in *mPer1* expression by cocaine in the NAc is not prevented in the *Npas2* mutant mice even though cocaine leads to an increase in NPAS2 binding at the *mPer1* promoter in this

region. *mPer1* and *mPer2* both have CREB binding sites in their promoters, however only *mPer1* seems to be responsive to CREB regulation (Travnickova-Bendova *et al.* 2002). Therefore, it is possible that the increase in *mPer1* expression in the NAc with cocaine treatment is due to both NPAS2 and CREB, and that CREB is sufficient to induce this activity in the absence of NPAS2. This is also supported by the fact that a dopamine D1, but not D2 type, antagonist can block the induction of *mPer1* in the CP by acute methamphetamine treatment (Nikaido et al., 2001) and a D1, but not D2, agonist can induce *mPer1* expression in striatal neurons (Imbesi et al., 2009). It is interesting that we find *mPer2* to be altered in the CP and not the NAc with chronic cocaine treatment. The NAc is associated with the regulation of drug reward and the processing of emotional stimuli, while the CP has been linked to the regulation of motor activity, as well as the control of habitual and compulsive behaviors (Nestler 2005, Wise 1998, Everitt & Robbins 2005). Thus PER2 may be more involved in behaviors controlled by the CP. Indeed previous studies have found that a line of mice lacking *mPer2* ^{Brdm1} show no differences in the conditioned place preference for cocaine, but have an increase in alcohol seeking behavior and self-administration, which may involve the CP (Abarca et al. 2002, Spanagel et al. 2005).

Alterations in clock gene expression in the striatum in response to drug treatment has profound behavioral effects on the animal in terms of altering the sleep/wake and activity cycle, as animals awake and increase their locomotor activity in anticipation of the drug (Iijima et al. 2002, Kosobud *et al.* 2007; Gillman

et al., 2008; Gillman et al., 2009). This anticipatory behavior is observed at least 2 hrs prior to the time of drug administration. Furthermore, changes in clock gene expression in reward related regions like the NAc may reinforce the importance of cocaine over other stimuli at certain times of day, and lead to an increased craving at the time when cocaine is expected. Thus, it was of interest to investigate the effect of chronic cocaine on the rhythmic expression of these genes in striatal regions. The purpose for this was two-fold. First, assess if the regulation already observed by cocaine was a timepoint-specific effect or if it was an overall change in the gene's rhythmic expression; and secondly, determine if there was an anticipatory effect in the expression of these genes that could presumably underlie the behavioral anticipation. Interestingly, cocaine treatment disrupted rhythmic expression of *Npas2* and led to an overall increase in *mPer1* and *mPer3* in the NAc. In the CP, chronic cocaine led to a disruption in the rhythmic expression of all the *Per* genes. *Clock* was relatively unaffected, supporting the idea that in striatal regions, Npas2, and not Clock, is a key player in mediating cocaine responses. A pilot study was performed to determine if there was any effect of cocaine in rhythmic expression of *mPer2* in the SCN. No such regulation was observed, save for a slight reduction in the peak of the rhythm (data not shown). The upregulation of certain genes at ZT 4 (2 hrs prior to administration time) despite the overall effects on rhythms suggest a possible anticipatory rise in circadian gene expression that could drive anticipatory locomotor events. It is important to note that this timepoint is 46 hrs after the animal's last cocaine injection, making this a persistent effect just like the one

observed behaviorally, which persists for a couple of days after drug has been withheld. It is, however, still unclear whether this increase in gene expression leads to the increased locomotor activity or vice-versa, since this is mostly correlative. For behavioral entrainment to occur, a manipulation or regulation of the circadian clock machinery must occur by photic and/or non-photic cues. Thus, it is likely that this alteration in core circadian clock genes in the striatum would in turn alter dopaminergic neurotransmission, which is known to be involved in locomotion and arousal. Therefore it is possible that these changes in core circadian gene expression underlie the increase in anticipatory locomotor activity observed in behavioral entrainment to drugs of abuse. It would be interesting to measure how long this effect lasts, since studies have shown that twice daily morphine injections for 7 d causes significant alterations in circadian gene rhythmic expression, as well as circadian patterns of hormones and peptides, that persist up to 60 d of withdrawal (Li et al., 2010). Our findings demonstrate that chronic cocaine regulates and disrupts the circadian system in striatal regions, suggesting a possible role of these regions as drug-entrainable oscillators.

CHAPTER FOUR

CIRCADIAN GENES IN THE NUCLEUS ACCUMBENS REGULATE COCAINE REWARD

Introduction

The majority of organisms display behavioral and physiological cycles with a duration of approximately 24 hours as an evolutionary response to the Earth's rotation on its axis. In mammals, the master clock that controls most of these activity rhythms is located in the Suprachiasmatic Nuclei (SCN) of the anterior hypothalamus and is primarily entrained by light (Reppert and Weaver, 2001). However, expression of core-clock circadian genes is not limited to this region, but is widespread throughout the brain and body (Hastings et al., 2003). The molecular clock is composed of interacting positive and negative transcriptional/translational feedback loops. The circadian locomotor cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL-1) proteins act as major transcriptional activators by forming a heterodimer that promotes transcription of the *Period* genes (*Per1, Per2,* and *Per3)*, the *Cryptochrome* genes *(Cry1* and *Cry2)*, as well as many other genes by binding to E-box elements (CANNTG) in their promoters (Reppert and Weaver, 2001). Following translation, the PER and CRY proteins go through post-translational modifications that promote their dimerization and nuclear entry in order to inhibit CLOCK-BMAL1 mediated transcription (Harms et al., 2003). In forebrain regions

or in conditions where CLOCK is nonfunctional, Neuronal PAS domain protein 2 (NPAS2), a protein similar in structure and function to CLOCK, can induce expression of the *Per* and *Cry* genes (Reick et al., 2001; Debruyne et al., 2006). Dysfunction of this circadian machinery has been implicated in a wide range of diseases and neuropsychiatric disorders, including drug addiction (Takahashi et al., 2008; Menet and Rosbash, 2011).

Recent studies have implicated the genes that make up the circadian clock in the behavioral responses to drugs of abuse. Pioneering studies performed in *Drosophila melanogaster* showed that flies lacking a functional *Period (Per), Clock, Cycle* or *Doubletime* gene all fail to sensitize to cocaine (Andretic et al., 1999). Later, Abarca *et al* found that *mPer1*^{Brdm1} knock-out mice fail to sensitize to cocaine and show no conditioned preference for cocaine, while *mPer2*^{Brdm1} knock-out mice show a hyper-sensitization to cocaine, but normal levels of conditioned preference for cocaine, suggesting a regulatory role for the *Per* genes in cocaine-associated behaviors (Abarca et al., 2002). However, a recent study by the same group found that the *mPer1*^{Brdm1} knock-out mice were indistinguishable from wild-type littermates in terms of their inclination to self-administer cocaine and reinstate their cocaine-seeking behavior, in clear contrast to their previous study (Halbout et al., 2010). Furthermore, the *mPer1*^{Brdm1} mice display normal levels of alcohol self-administration and reinstatement of alcohol-seeking behavior, while the *mPer2*^{Brdm1} mice show an increase in alcohol self-administration (Zqhoul et al., 2007; Spanagel et al., 2005). Studies have also shown that morphine reward is regulated by the *Per* genes. *mPer2*^{Brdm1} mutant

mice were found to fail to develop tolerance to the analgesic effects of morphine when tested on hot plate or tail-immersion tests (Perreau-Lenz et al., 2010). Moreover, these mice also showed a suppression of morphine-induced withdrawal signs when compared to their wild-type counterparts (Perreau-Lenz et al., 2010). The gene *mPer1* has also been implicated in regulating the rewarding effects of morphine (Liu et al., 2005). A reduction in *mPer1* by use of a DNAzyme was found to block conditioned preference for morphine, which may involve the ERK signaling pathway (Liu et al., 2005; Liu et al., 2007). Thus, both *mPer1* and *mPer2* are involved in regulating the behavioral and physiological responses to morphine.

Previous work from our laboratory reported that mice carrying a mutation (Δ19) in the *Clock* gene show hyperactivity, an increase in the reward value for cocaine both in conditioned place preference and intracranial self-stimulation paradigms, that is associated with an overall behavioral state that resembles human mania (McClung et al., 2005; Roybal et al., 2007). NPAS2 shares a highly similar primary amino acid sequence and function with CLOCK and both can drive the circadian loop (Zhou et al., 1997; Reick et al., 2001). However, while CLOCK is widely expressed throughout the brain, including high levels in the SCN, the expression of NPAS2 is restricted to the forebrain, with no expression in the SCN (Garcia et al., 2000; King et al., 1997; Zhou et al., 1997). Previous reports have found that mice lacking a functional NPAS2 protein have defects in cued and contextual fear conditioning, altered patterns of sleep, and are unable to properly entrain to daytime food restriction, suggesting that NPAS2 has

important functions in the brain (Dudley et al., 2003; Garcia et al., 2000). Since NPAS2 has little to no expression in the VTA, but is highly enriched in the striatum, especially in the NAc, which receive input from midbrain dopaminergic regions, NPAS2 may also be involved in regulating the response to drugs of abuse.

This study further demonstrates the role for circadian genes in regulation of drug reward. As an extension to the previous work done on Clock mutant mice, other lines of circadian mutants were tested for cocaine preference. Additionally, specific knockdown of circadian genes was localized to the NAc, a region known to be involved in the rewarding effects of drugs of abuse, as a way to narrow down the regions where these genes might act in their regulation of reward.

Materials and Methods

Animals

Npas2 (B6/129S6) mutant mice (Garcia et al., 2000) were generously provided by Steve McKnight (UTSW). *mPer1, mPer2,*and *mPer1/mPer2* (129S4) mutant mice (Bae et al., 2001) were generously donated by David Weaver and colleagues (UMass). Wild type littermate controls were utilized in all studies of single gene knock-outs. Age and sex matched wild-type littermates from the *mPer1* and *mPer2* lines were used as controls for the *mPer1/mPer2* mutant mice. Wild-type C57BL/6J mice were purchased from Jackson Laboratories for all gene knockdown studies. All animals were group housed in a 12/12 light/dark

cycle (lights on 7am, lights off 7pm) with food and water *ad libitum*. Male mice between 6-8 weeks old were used in all studies, and all measures were performed between ZT3-6. All animal use was approved by our Institutional Animal Care and Use Committee.

Drug

Cocaine Hydrochloride (HCl) was generously provided by the National Institute on Drug Abuse. Animals were injected with different doses of cocaine for CPP experiments. Doses used were 2.5, 5.0 and 10.0 mg/kg ip.

Conditioned Place Preference (CPP)

An unbiased conditioning protocol, based on published methods (McClung and Nestler, 2003), was used. Briefly, male mice 6-8 weeks old were habituated in the testing room for 30 min to 1 hr before testing or conditioning. Mice were tested for 20 min in the place-conditioning apparatus before conditioning on day 1 to ensure there was no bias toward any chamber of the apparatus. Mice that spent >15 min in any one compartment before conditioning were discarded from the study (this accounted for <10% of the total animals). On days 2 and 4, mice were given a saline injection paired with one side of the chamber of the apparatus, and on days 3 and 5, mice were given a cocaine injection paired with the other side chamber of the apparatus. Each conditioning session lasted 20 min, and sessions were conducted at the same time of day

(ZT3-5). On day 6, mice were assayed for the time spent in the two side chambers of the apparatus. Data was analyzed by Student's T-test.

Locomotor response to novelty

Mice were individually placed in home-cage environment inside automated locomotor activity chambers equipped with infrared photobeams (San Diego Instruments, San Diego, CA) and measurements began immediately. Activity of the animal was continuously measured and the data was collected in 5-min blocks over a period of 2 hours.

Construction of shRNA constructs and viral packaging

A small hairpin RNA (shRNA) was constructed against *mPer1* and *mPer2* mRNA by selecting a conserved 24 base sequence (5'- ATCCCTCCTGACAAGA GGA TCTTC-3') in the coding region (See Appendix p. 127, Figure A.4, for knockdown efficacy *in vivo* of the *mPer1;mPer2* shRNA). For the *Npas2* gene, an shRNA was constructed by selecting a 24 base sequence (5'-GAACACTG GATTCTTCCTGT TAAC -3') in the 3'-UTR. The *Clock* shRNA was previously published by members of the laboratory (Mukherjee et al., 2010). For the scrambled (Scr) shRNA, a random sequence of 24 bases (5'-CGGAATTTAGTTACG GGGATCCAC-3') that had no sequence similarities with any known genes/mRNA was used. An antisense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotides (CTTCCTGTCA) was added at the 5'end of the above sequences. The miR23 loop facilitates the

transfer of the hairpin RNA out of the nucleus. The constructs were first tested *in vitro* on mouse embryonic fibroblast cultures. Constructs were transfected using Lipofectamine LTX Reagent (Invitrogen) according to manufacturer's instructions. These shRNAs were designed as synthetic duplexes with overhang ends identical to those created by Sap I and Xba I restriction enzyme digestion. The annealed oligonucleotides were cloned into the adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (Stratagene, La Jolla, CA). Plasmids were sent to the University of North Carolina Gene Therapy Viral Vector Core for packaging into adeno-associated viral (AAV) vectors (Chapel Hill, NC).

Stereotaxic surgery

Stereotaxic surgery was performed similarly to Mukherjee et al 2010. Mice were anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline (0.9% NaCl). Bilateral stereotaxic injections of 1 µl of purified high titer AAV encoding scrambled or AAV- shRNA was injected into the NAc (from bregma: angle 10°, AP +1.5 mm, Lat +1.5, DV -4.4) using a 33 gauge hamilton syringe (Hamilton, Reno, NV). Injection speed was 0.1 µl/minute, and the needle was kept in place for an additional 5 minutes before it was slowly withdrawn. Mice recovered for two weeks in their home cage prior to behavioral testing to allow for full virus expression. A separate group of mice was used to measure the efficacy of knockdown *in vivo* for the

Npas2shRNA virus. Mice were injected in the NAc, and tissue was processed for RNA and real time RT-PCR analysis.

Immunohistochemical localization of AAV expression

Mice were anesthetized with 50 mg/kg Nembutal in saline, and transcardially perfused with 4% paraformaldehyde in 1X PBS (1 mM KH2PO4, 10 M Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The brains were allowed to post-fix in a 4% paraformaldehyde for 24 hours and then placed in 1X PBS-30% glycerol sucrose protection for an additional 24 hours before being stored in 1X PBS-0.05% sodium azide. 30 µm brain sections were obtained with a microtome (Leica, Wetzlar, Germany) and immunohistochemical staining against GFP (AbCam, Cambridge, MA) was carried out using standard procedures . Secondary antibodies (anti-rabbit conjugated with Alexa 488) was purchased from Molecular Probes (Carlsbad, CA). Brain sections were mounted using Vectashield (Vector Labs, Burlingame, CA) with DAPI counterstaining and observed with an epifluorescence microscope with a 10x objective. Animals were excluded from our study if their infection spread was not localized to the NAc , with spillover to adjacent areas or throughout the injection tract; or if there was a significant disproportionate amount of infection between both hemispheres. Exclusion by these criteria accounted for approximately 10% of animals.

Statistical analysis:

All data are expressed as mean ± standard error of the mean. One-way ANOVA was used to analyze group differences in behavior where applicable. Two-way ANOVAs were used to analyze differences among groups in the locomotor activity assays. Behavioral results from the shRNA experiments were analyzed by student's t-test. In all experiments $P<0.05$ is considered significant.

Results

Npas2 mutant mice show a decrease in the conditioned preference for cocaine

To determine if NPAS2 is important in regulating behavioral measures associated with cocaine reward, we employed mice that have a disruption in the PAS domain of *Npas2* (Generated by Garcia, Mcknight and colleagues) (Garcia et al., 2000). This mutation leads to a nonfunctional protein. In contrast to the *Clock* mutant mice which are extremely hyperactive (McClung et al., 2005b), the *Npas2* mutant mice have normal baseline locomotor activity (data not shown). Furthermore, in contrast to the *Clock* mutant mice, the *Npas2* mutant mice have a marked decrease in their conditioned preference for cocaine (Figure 4.1). This decrease was observed at the low doses of 2.5 and 5.0 mg/kg, but not at 10 mg/kg, suggesting a ceiling effect at this dose.

Effects of *Npas2* knockdown in the NAc

To determine efficacy of *Npas2* knockdown, mice were injected in the NAc, allowed to recover, and tissue was processed for real-time RT-PCR.

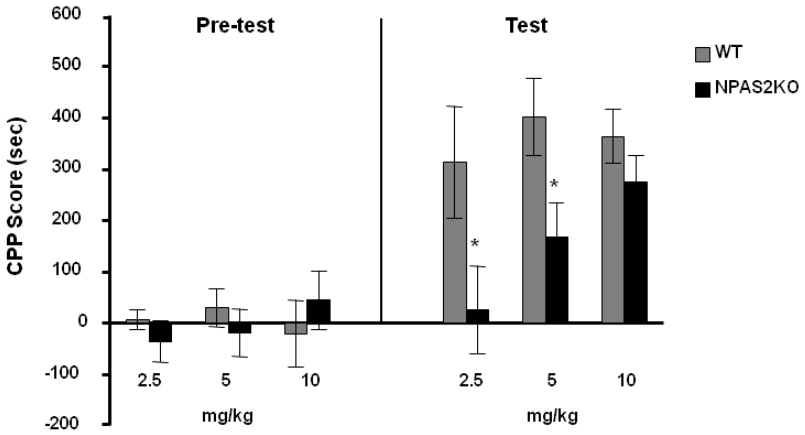

Figure 4.1 Cocaine conditioned place preference in the *Npas2* mutant mice. *Npas2* mutant mice exhibited a decreased cocaine preference when compared to their wild-type counterparts at 2.5 and 5.0 mg/kg of cocaine. Tests were performed using an unbiased protocol at 2.5, 5, and 10 mg/kg cocaine. Data is mean±SEM. *P < 0.05 by t-test, n=25-30.

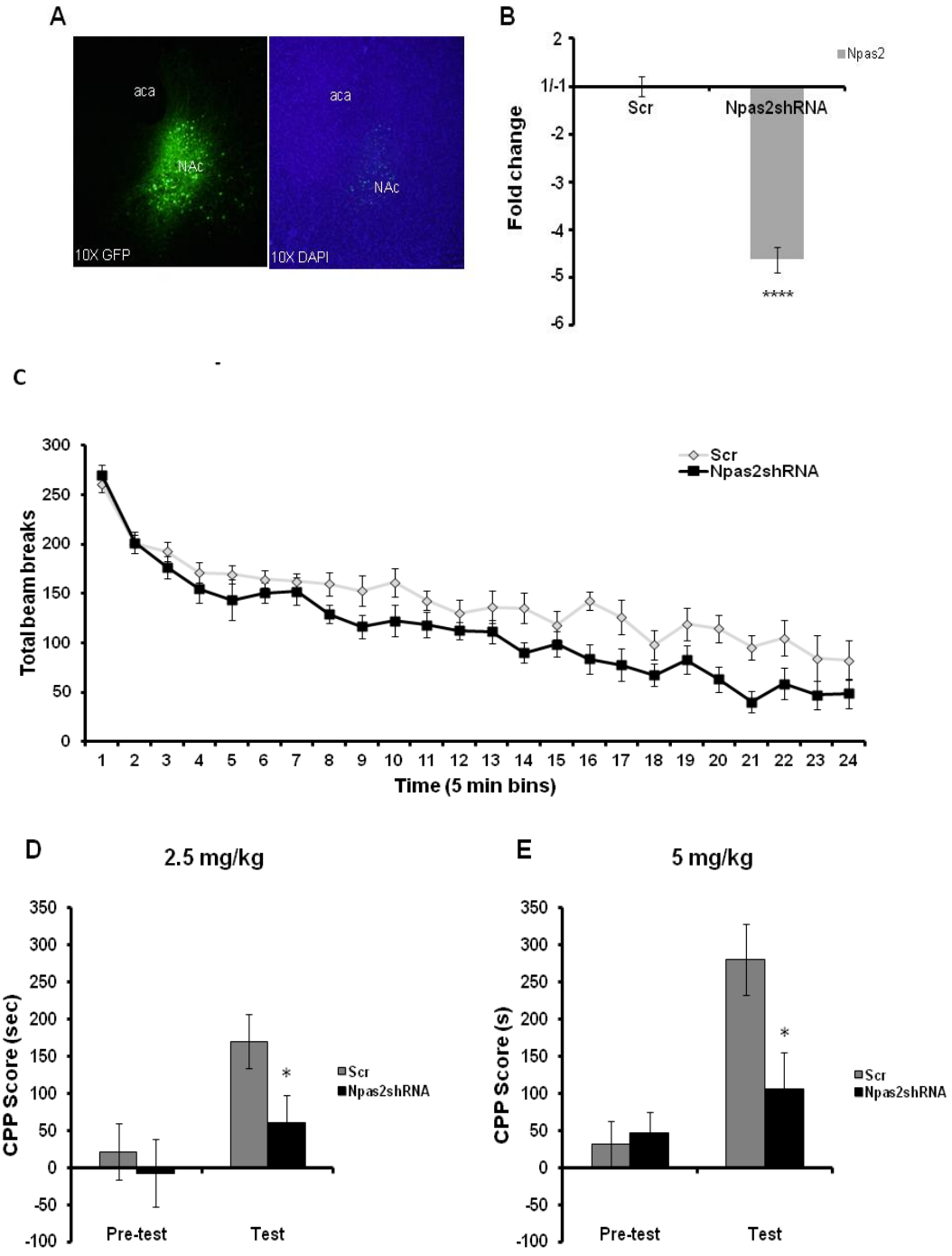
A
aca
NAc
10X GFP
aca
NAc
10X DAPI
B
Npas2
Scr
Npas2shRNA
Fold change
****
C
Scr
Npas2shRNA
Total beam breaks
Time (5 min bins)
D
2.5 mg/kg
CPP Score (sec)
Scr
Npas2shRNA
Pre-test
Test
E
5 mg/kg
CPP Score (s)
Scr
Npas2shRNA
Pre-test
Test

Figure 4.2 *Npas2* knockdown in the NAc. (A) Representative image showing viral expression in the NAc. (B) AAV-Npas2shRNA infusion leads to a significant knockdown of Npas2 expression in the NAc. (C)Localized knockdown in the NAc causes a significant decrease in locomotor activity that is noticeable during the second hour of the locomotor test ($F_{(1,408)}$=55.57, p<0.0001 treatment effect). (D,E) Npas2shRNA injected animals displayed a decrease in cocaine preference, similar to the NPAS2KO mice. (Data is mean±SEM. *P < 0.05 by t-test, n=9-10).

Figure 4.2A shows the localization of a viral infection in the NAc at 10x magnification and stained for GFP and DAPI. Infection with the AAV-*Npas2*shRNA virus in the NAc of intact animals led to a ~5-fold decrease of *Npas2* mRNA compared with controls (Figure 4.2B, ***p<0.0001). Localized knockdown of *Npas2* in the NAc had an effect in locomotor activity response (Figure 4.2C). Npas2-shRNA injected mice displayed a significant reduction in locomotor activity that was only noticeable during the second hour of the test, suggesting an enhanced habituation to the environment over time ($F_{(1,408)}$=55.57, p<0.0001 treatment effect). A reduction in *Npas2* expression in the NAc was sufficient to cause a significant decrease in cocaine preference at both cocaine doses, mimicking the effect observed in the NPAS2 mutant mice (Figure 4.2 D, E). The decreased locomotor response to novelty over time does not confound the interpretation of CPP measures since CPP sessions last, at most, 30 min. At 30 min, there is no significant difference in locomotor activity.

Effects of *Clock* knockdown in the NAc

Since the *Clock* mutant mice showed both a hyperactivity and increased preference for cocaine, the effect of the gene knockdown on locomotor activity and preference was initially measured. AAV-Clock shRNA was found to significantly reduce Clock expression (Mukherjee et al., 2010; Figure 4.3A). Viral-mediated knockdown of *Clock* in the NAc of wild-type mice had no effect in locomotor activity (Figure 4.3B). *Clock*-shRNA injected mice were indistinguishable from Scr-injected mice.

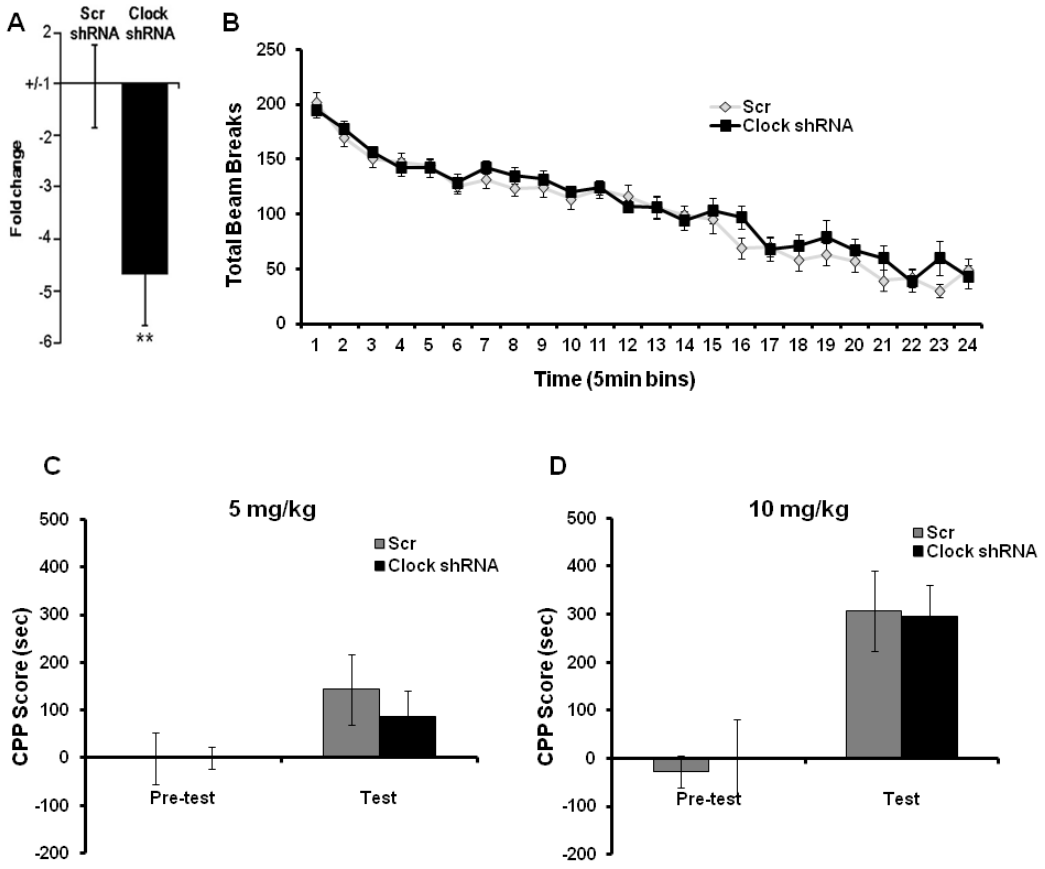

Figure 4.3 *Clock* knockdown in the NAc. (A) Clock shRNA leads ro a significant reduction in *Clock* expression. Panel is taken directly from Mukherjee et al., 2010. (B) Clock-shRNA injected mice showed similar locomotor activity levels as control mice. (C,D) No difference in cocaine preference was observed between Clock-shRNA injected mice and control mice under both doses of cocaine. Data is mean±SEM (n=8-14 for locomotor assay; n=6-9 for CPP).

Moreover, *Clock* knockdown in the NAc had no effect on cocaine CPP measures (Figure 4.3C, D). Mice injected with the *Clock*-shRNA were indistinguishable from the Scr-injected mice at both doses, developing a normal preference for cocaine. Thus, these data suggest that proper CLOCK function in the NAc is not necessary for a proper locomotor response to novelty or the conditioned preference for cocaine.

A mutation in *mPer1;mPer2* leads to an increase in the preference for cocaine

The PER proteins are involved in a negative feedback loop that reduces the activity of NPAS2. Therefore, removing the function of these genes could lead to opposing behavioral responses to those seen in the *Npas2* mutant animals. To determine if mice lacking the PER proteins have a change in the behavioral responses to cocaine, we tested mice lacking functional *mPer1*, *mPer2*, or *mPer1* and *mPer2* (generated by Weaver, Reppert and colleagues)(Bae et al. 2001). Similar to the *Npas2* mutants, these mice have a normal locomotor response to novelty (data not shown). A significant increase in cocaine preference was observed in mice with mutations in either *mPer1* or *mPer2* over wild type mice, however, the *mPer1;mPer2* double mutant mice displayed a much more robust increase in their preference for cocaine (Figure 4.4). This suggests that *mPer1* and *mPer2* have opposing actions to NPAS2 in the regulation of cocaine reward.

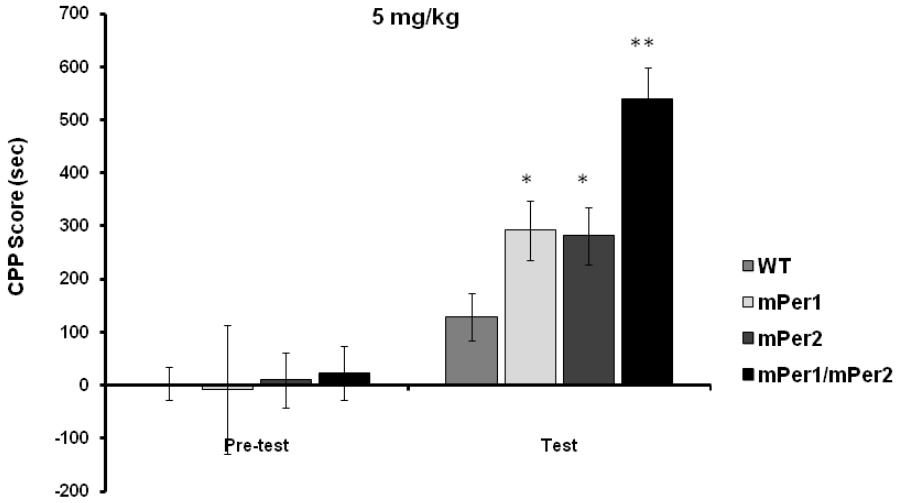

Figure 4.4 Effects of the *Per* mutations on the preference for cocaine. Single mutant mice displayed an increase in cocaine preference, whereas the double mutant mice displayed an even greater increase in cocaine preference. **P < 0.01 by ANOVA, *P<0.05. Individual mutants were compared with their own littermate controls. Graphed is an average of the wild type response which did not differ significantly among lines. n=20-30.

Effect of *mPer1/mPer2* knockdown in the NAc

Since the mPer1/mPer2 double mutant mice exhibited such a robust increase in cocaine preference, a shRNA construct was constructed to allow knockdown of both genes simultaneously in the NAc. This virus has been tested and used in other studies (Appendix p.127, Figure A.4). Knockdown of both *mPer1* and *mPer2* in the NAc led to a significant decrease in locomotor activity, consistent with increased habituation that was noticeable only during the second hour of the locomotor assay (Figure 4.5A). When tested for cocaine preference, there was no significant difference between shRNA and scr injected mice at the 2.5 mg/kg dose (Figure 4.5B). A trend towards an increase in preference was observed at the 5 mg/kg dose.

Discussion

This study found that NPAS2 and the PER proteins are involved in the regulation of cocaine reward. Though NPAS2 and CLOCK are very similar proteins, mutations in each lead to opposite effects on cocaine preference. This suggests that these proteins have different functions in the pathways that regulate reward. It is also possible that the effects of these genes on these measures occur through completely independent pathways or in different brain regions, as this work suggests. In fact, regulation of drug reward by Npas2 seems to be via actions in the NAc, since localized knockdown of Npas2 in this region was sufficient to recapitulate the decreased preference phenotype

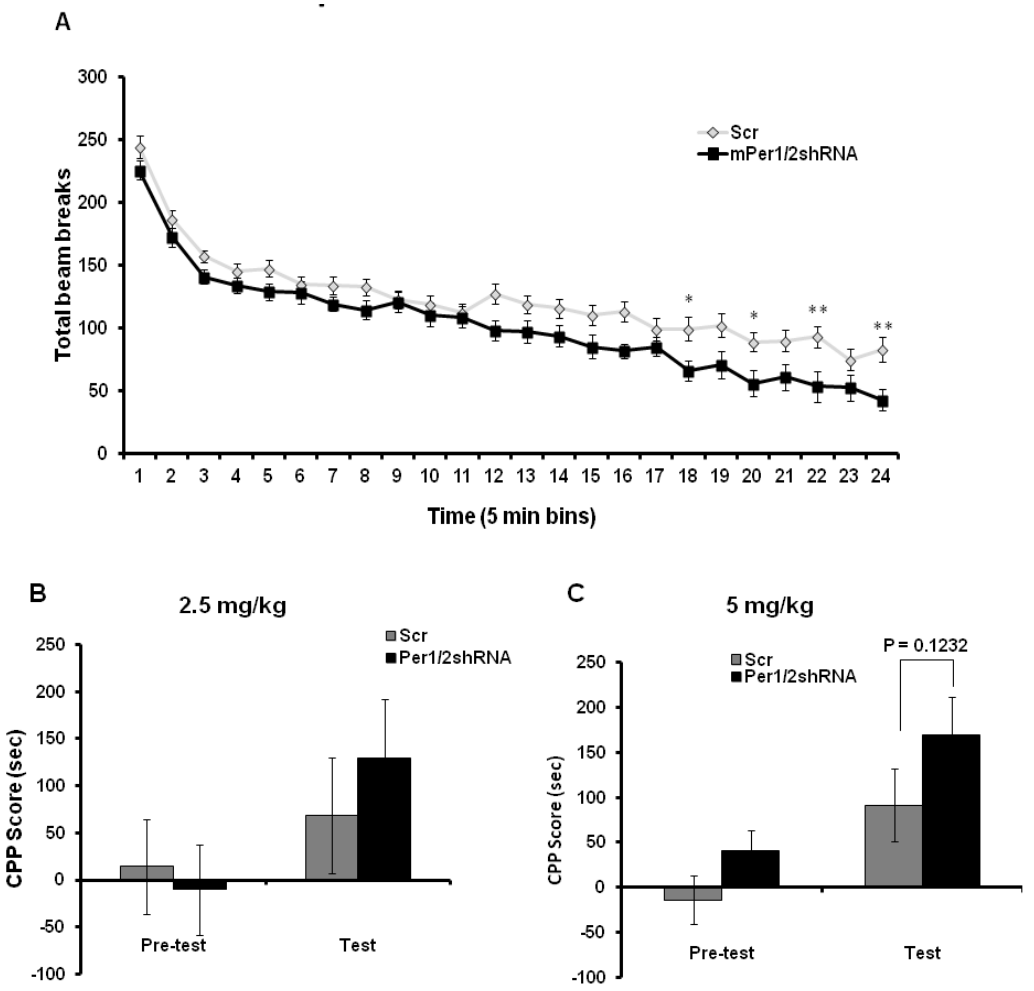

Figure 4.5 *mPer1/mPer2* knockdown in the NAc. (A) Localized knockdown in the NAc causes a significant decrease in locomotor activity that is noticeable during the second hour of the locomotor test ($F_{(1,1032)}$=94.98, p<0.0001 treatment effect). (B,C) No difference in cocaine preference was observed between Per1/2-shRNA injected mice and control mice under both doses of cocaine. A trend towards an increase in cocaine preference is observed at the 5mg/kg dose (C; p=0.1232). Data is mean±SEM (n=16-22 for locomotor assay; n=10-12 for CPP). See Appendix p.127, Figure A.4 for viral knockdown efficacy.

observed in the NPAS2 mutant mice. The fact that *Clock* knockdown in the NAc produced no effect in cocaine preference is perhaps not surprising. Previous studies have established that CLOCK's actions in the Ventral Tegmental Area (VTA) are important for the regulation of dopaminergic activity, as well as manic and depressive-like behavior (Roybal et al., 2007; Mukherjee et al., 2010). Thus we believe that CLOCK's actions on reward-related behavior are primarily in the VTA, while NPAS2 primarily acts in the NAc, where it is highly expressed.

In an effort to understand the differences in behavior observed with the *Npas2* mutant mice, a microarray was performed comparing NAc tissue from these mice to wild type mice. Several genes were found to be differentially regulated in this region in the *Npas2* mutant mice versus wild type littermates, however, what was striking was that a number of genes that were upregulated in the NAc of the *Npas2* mutant mice (10 genes out of 25 total, including the top eight most highly regulated genes) were also identified in previous studies to be upregulated in the NAc of mice overexpressing the transcription factor, CREB (unpublished observations; McClung and Nestler, 2003). In addition, most of these genes were also found to be upregulated in the NAc following a 5 day chronic cocaine treatment (McClung and Nestler, 2003). Interestingly, CREB phosphorylation at Ser133 is increased selectively in the NAc of the *Npas2* mutant mice (unpublished observations). This increase presumably is responsible for the increase in a number of CREB target genes that we find in the NAc of these mice (unpublished observations; McClung and Nestler, 2003). Furthermore, the *Npas2* mutant mice display a decrease in cocaine preference

that is very similar to the behavioral responses seen in these measures in mice or rats that overexpress CREB in the NAc either through viral-mediated gene transfer or transgenics (Barrot et al., 2002; Carlezon et al., 2005; McClung and Nestler, 2003). Expression of a dominant negative CREB in the NAc has the opposite effect on these behavioral measures and on gene expression (Barrot et al., 2002; Carlezon et al., 2005; McClung and Nestler, 2003). Therefore, it is possible that the behavioral effects that are observed in these mice are due to the increase in CREB activity that occurs in the NAc. It is important to note that all of these behavioral observations have been measured via CPP, including this present study. However, an increase in CREB in the NAc leads to increased cocaine reinforcement as measured by self-administration (Larson et al., 2011). Thus, these two different methods of drug administration can lead to different behavioral outcomes and an extension of our studies to a self-administration paradigm is important. How a reduction in NPAS2 leads to an increase in CREB is unclear.

P-CREB levels are known to have a circadian rhythm in the SCN that is presumably controlled by the actions of the circadian genes (Ginty et al., 1993). Our data suggests that a similar regulation of P-CREB by circadian genes may occur in the NAc. It will be interesting in future studies to determine if NPAS2 is involved in regulating components of the protein kinase A or other signal transduction pathways involved in the phosphorylation of CREB.

Mice lacking a functional *mPer1* and *mPer2* show an opposite behavioral response to that of the *Npas2* mutant mice in measures of cocaine preference.

This is perhaps not surprising since the PER proteins are involved in negatively regulating the activity of NPAS2 so a lack of *Per* gene function should result in an increase in NPAS2 regulated genes. A significant increase was observed in measures of cocaine preference in mice lacking *mPer1*or *mPer2* individually. This is in contrast to a study published previously showing that mice lacking *mPer1*^{Brdm1} have a decrease in cocaine preference (Abarca et al., 2002). It is unclear why there is a difference in behavior between these two lines, but it could be due to differences in strain or testing paradigms. These two mouse lines do display marked differences in their circadian locomotor phenotypes when measured in constant darkness (Bae et al., 2001; Zheng et al., 2001). The fact that mutations in *mPer1*and *mPer2* together leads to such a dramatic change in behavior when compared to the individual mutants suggests that these two genes have some overlapping and compensatory functions in regard to the regulation of drug reward. This is similar to the effects on circadian locomotor rhythms in these mice in that the double mutations produce a much more severe defect in rhythms than either of the single mutations when mice are kept in constant darkness (Bae et al., 2001). Knockdown of *mPer1* and *mPer2* in the NAc resulted in a trend towards increased preference and not a significant effect. This highlights some of the limitations of this technique. The major limitation is that it is well known that viral-mediated knockdown does not result in a reduction of the desired protein as complete as a constitutive knockout animal. Additionally, the amount of viral expression and spread is variable from surgery to surgery. These factors could in turn dilute the effects observed. Additionally, even though

mPer3 was the most regulated by cocaine when compared to the other Per genes (Figure 3.6), we decided to focus on core circadian clock genes, as Mper3 seems more important in peripheral body organs than in the central nervous system (Pendergast, 2012).Interestingly, the *mPer1;mPer2* mutants have a decrease in P-CREB levels in the NAc (unpublished observations). Therefore, the opposing behavioral responses seen in these mice as compared to the *Npas2* mutants correlates with opposing levels of P-CREB. Taken together, these findings support an important and distinct role for NPAS2 and the PER proteins in striatal regions in the regulation of reward that likely involves the modulation of P-CREB.

CHAPTER FIVE

POTENTIAL DIRECT CIRCADIAN CONTROL OF DOPAMINERGIC RECEPTORS IN THE NAC AND THEIR REGULATION BY COCAINE

Introduction

Disruptions in circadian rhythms are thought to contribute to the pathophysiology of several psychiatric diseases, including drug addiction (Menet and Rosbash, 2011). These rhythms are controlled primarily by transcriptiona/translational feedback loop in the Suprachiasmatic Nucleus (SCN) of the anterior hypothalamus (Reppert and Weaver, 2001). Even though the master pacemaker is located in the SCN, circadian genes and proteins are widely expressed throughout the brain, thereby forming SCN-independent pacemakers that entrain to other non-photic stimuli such as food (Iijima et al., 2002; Stephan, 1984). Drugs of abuse can also serve as powerful Zeitgebers for some of these clocks outside of the SCN. Several studies have shown that drugs of abuse, like cocaine, methamphetamine, nicotine and alcohol, can entrain locomotor activity rhythms (Kosobud et al., 2007). Additionally, behavioral responses and sensitivity to drugs of abuse, especially psychostimulants, exhibit diurnal variations (Manev and Uz, 2009).

The diurnal variations observed in mood, reward, affective disorders, and responses to drugs are thought to arise from an interaction and crosstalk

between the circadian system and multiple neurotransmitter systems. In fact, a number of neurotransmitters, like dopamine, glutamate and GABA show circadian rhythms in their release in the striatum and nucleus accumbens of awake rats (Castañeda et al., 2004). Both the circadian system and the serotonergic system are important in mediating affective and temporally gated behaviors as well as influencing development of brain circuits involved in biological rhythms and affective behaviors (Ciarleglio et al., 2011). Many of the components of the serotonergic system exhibit a diurnal variation, not just in the midbrain raphe nucleus, but also in the SCN (Cagampang and Inouye, 1994; Malek et al., 2005). The rate limiting enzyme for serotonin synthesis, TPH, its message *tph2*, and the secretion of serotonin itself exhibit circadian rhythms in the midbrain (Malek et al, 2005, 2007). Additionally, the serotonin transporter, SERT, also shows diurnal expression in the brain, which is the reason why behavioral effects to antidepressant selective serotonin reuptake inhibitors (SSRIs) display a diurnal variability (Ushijima et al., 2005). The dopaminergic system is also heavily regulated by the circadian system. In fact, virtually all elements involved in dopaminergic transmission display diurnal variability (McClung, 2007). The rate limiting enzyme for dopamine synthesis, TH, is known to exhibit a circadian rhythm in striatal regions (Sleipness et al., 2007). Both the dopamine transporter (DAT) and TH show higher expression during the dark phase in the NAc, whereas TH, and not DAT, also shows variation in the CP (Sleipness et al., 2007). These diurnal effects were not observed in SCN-lesioned rats, suggesting a role for the SCN in maintaining this rhythmicity.

Moreover, dopamine receptor expression as well as response to quinpirole, a D2/D3 receptor agonist, has also been shown to be rhythmic (Akhirasoglu et al., 2005). This suggests that variation in dopamine receptor expression could account for dosing time-dependent changes in quinpirole-induced locomotor behaviors or dopamine-mediated behaviors. However, the mechanism underlying this variation in dopaminergic expression remains unknown. It is unclear whether this is a direct target effect of Clock or Npas2 or if it is being mediated indirectly via other clock-controlled genes. This study determined whether Clock or Npas2 can bind directly the promoter regions of dopaminergic receptors *DRD1, DRD2,* and *DRD3* in the striatum, and whether their diurnal expression in the NAc is mediated by Npas2. Additionally, just how cocaine can disrupt rhythmicity of circadian genes in the NAc (Chapter Three), this study assessed the effect of chronic cocaine treatment on rhythmic expression of dopaminergic receptors in the NAc.

Materials and Methods

Animals

C57BL/6 mice (The Jackson Laboratory) were group housed in a 12/12 light/dark (LD) cycle (lights on at 7am, lights off at 7pm) with food and water *ad libitum*. For the 24-hr time series studies, mice were group housed under the same LD schedule in temperature-controlled and sound-proof cabinets. Male mice between 6-8 weeks old were used in all studies. All animal use was approved by the UTSW Institutional Animal Care and Use Committee.

Drug

Cocaine Hydrochloride (HCl) was generously provided by the National Institute on Drug Abuse. Animals were injected with a 15mg/kg cocaine or saline i.p. in all studies.

Real time RT-PCR

RNA was isolated from mechanically homogenized tissue using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. This was followed by a 15 min treatment with DNAse I (Invitrogen) according to manufacturer's protocols to digest any remaining genomic DNA. One microgram of total RNA was used to synthesize cDNA using Superscript III Reverse Transcriptase (Invitrogen) per the manufacturer's instructions. cDNA or chromatin samples were mixed with SYBR Green master mix (Applied Biosystems, ABI) and specific primers for genes or promoter regions of interest (for a list of primer see supplemental materials). Prior to the experiment primer sets were tested thoroughly to determine reaction efficiency, specificity, and the absence of primer-dimers. Reactions were run on an ABI Prism 7700 real-time PCR machine. Fold changes and relative gene expression were calculated using the comparative Ct method and normalized to the corresponding *Gapdh* or *Cyclophilin* mRNA levels. The Ct values used for these calculations are the mean of at least four biological replicates of the same reaction; each PCR reaction was done in duplicate and used 5µl ofcDNA.

Chromatin Immunoprecipitation (ChIP)

Brain tissue was processed as previously reported (Enwright et al., 2010). Brain punches were taken in NAc and CP regions from 8 mice and pooled, and immediately cross-linked in 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125M. The tissue was washed 5 times in cold PBS containing Complete Protease Inhibitor Cocktail (Roche) and then frozen on dry ice. The chromatin was solubilized and extracted by detergent lysis, followed by sonication. First, fixed tissue was homogenized twice, for 10 sec, in a cell lysis buffer (10mM Tris, 10mM NaCl, 0.2% Nonidet P-40). Next, the extracted chromatin was sheared to roughly 500-1,000 bp using the Sonic Dismembrator 550 (Fisher, Hampton, NH). Each sample was sonicated 8 times on ice, 20 sec each, at 25% of maximum power. After the chromatin lysate was extracted and properly fragmented, the optical density of each sample was determined. Equal amounts of chromatin lysate, 60 µg, were diluted with ChIP dilution buffer (Upstate) to a final volume of 1.1 ml. 100 µl of the pre-immunoprecipitated lysate was saved as “input” for later normalization. The chromatin solution was pre-cleared with either salmon sperm DNA/protein A-agarose gel slurry (Thermo Scientific) or Protein G Agarose/Salmon Sperm DNA (Thermo Scientific) for 45 min at 4°C. It was then immunoprecipitated overnight at 4°C with an antibody directed against a specific protein either CLOCK H-276 X or NPAS-2 H-20 X (Santa Cruz Biotechnology, Santa Cruz, CA). As a control, samples were immunoprecipitated with non-

immune rabbit IgG (Upstate) or Anti-acetyl-Histone H3 (Upstate). Following immunoprecipitation, the DNA-protein complex was collected with either 40µl salmon sperm DNA/protein A-agarose beads or 50µl Protein G Agarose/Salmon Sperm DNA for 2 hr. The beads were sequentially washed once with low salt, high salt, LiCl, and twice with TE buffers. The DNA-protein complex was then eluted from the beads with 500 µl $NaHCO_3$/SDS elution buffer. Proteins were reverse-cross linked from DNA using Proteinase K (Invitrogen) under high-salt conditions at 65°C for at least 4 hr. The DNA, associated with a particular transcription factor, was extracted with phenol/chlorophorm/isoamyl alcohol, precipitated with 100% ethanol, washed with 70% ethanol, and finally resuspended in 0.1X TE Buffer diluted in PCR-grade water. Levels of specific transcription factor binding or histone modifications at each gene promoter of interest were determined by measuring the amount of associated DNA by real-time PCR (Applied Biosystems (ABI) Prism 7700, Foster City, CA). Input or total DNA (nonimmunoprecipitated) and immunoprecipitated DNA were amplified in duplicate in the presence of SYBR Green (ABI). Relative quantification of template DNA was performed using the comparative Ct method.

Stereotaxic surgery

Stereotaxic surgery was performed similarly to Mukherjee et al 2010. Mice were anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline (0.9% NaCl). Bilateral stereotaxic injections of 1 µl of purified high titer AAV encoding scrambled or AAV-Npas2 shRNA was injected into the NAc (from bregma: angle 10°, AP +1.5 mm, Lat

+1.5, DV -4.4) using a 33 gauge hamilton syringe (Hamilton, Reno, NV). Injection speed was 0.1 µl/minute, and the needle was kept in place for an additional 5 minutes before it was slowly withdrawn. Mice recovered for two weeks in their home cage to allow for full viral expression.

Collection of AAV- Npas2-shRNA infected tissue

Animals were euthanized at two different timepoints (ZT 4 and ZT 16) and brains were immediately frozen in dry ice and stored at -80°C. Brains were sliced in a cryostat with a thickness ranging from 30µm – 300µm. 30µm sections were immediately fixed and dried to observe localization of GFP expression using an epifluorescence microscope. Following identification of correct viral injection targeting, 150µm – 300µm sections were used for taking punches of NAc tissue, which were later processed for real-time RT-PCR.

Data analysis

ChIP assays were analyzed by one-sample t-tests, whereas changes in dopamine receptor expression were analyzed by t-test (*$p<0.05$). For the time series experiments, one-way ANOVAs followed by Tukey's Multiple Comparison Test were used to establish diurnal rhythmicity for each gene and each condition, as previously published (Maywood et al., 2010). Additionally, two-way ANOVAs followed by post-hoc t-tests were used to assess the overall effect of treatment and time.

Results

CLOCK and NPAS2 can directly bind to promoter regions of dopaminergic receptors in the striatum

To determine whether CLOCK or NPAS2 could bind promoter regions of dopaminergic receptors, animals were euthanized at night (ZT 16), when peak expression levels of dopaminergic receptors in the striatum are observed and whole striatum tissue was processed for ChIP assays. CLOCK was found to directly bind all three dopamine receptor promoters, whether NPAS2 was only bound to *DRD1* and *DRD3* promoter regions (Figure 5.1; *p<0.05, **p<0.01, ****p<0.0001).

Npas2 knockdown in the NAc alters diurnal expression of dopaminergic receptors

Since we found in the previous chapter that NPAS2 and not CLOCK, is a key player in mediating cocaine's responses in the NAc, its role in mediating dopaminergic rhythmic expression was assessed. Following viral-mediated knockdown of *Npas2* in the NAc, expression of dopaminergic receptors at two timepoints (ZT 16 (night) and ZT4 (day)) was measured by real time RT-PCR. Interestingly, *Npas2* knockdown specifically in the NAc abolished diurnal expression of all three dopamine receptor genes (Figure 5.2 A, B, C; *p<0.05, ***p<0.001).

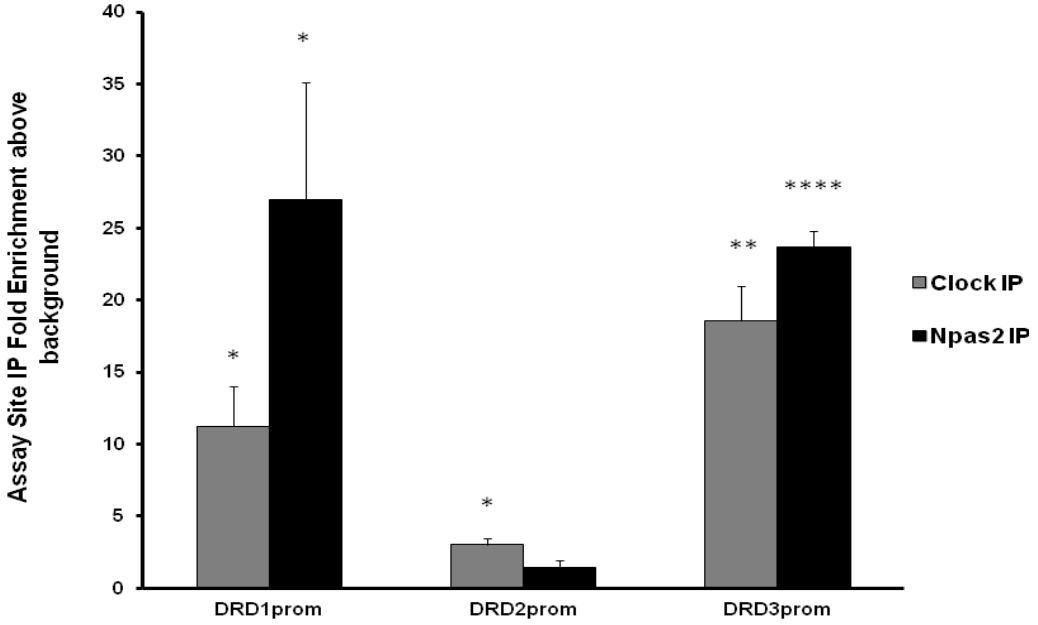

Figure 5.1 Clock and Npas2 can bind at promoter regions of dopamine receptors. A ChIP assay was performed at a time of day when mRNA expression levels of DA receptors are at their peak. CLOCK binds all three receptor promoters, whereas NPAS2 only binds DRD1 and DRD3 promoters. Data is mean fold ±SEM above IgG background. (*p<0.05, **p<0.01, ****p<0.0001, IP fold vs 1-fold; n=5-7).

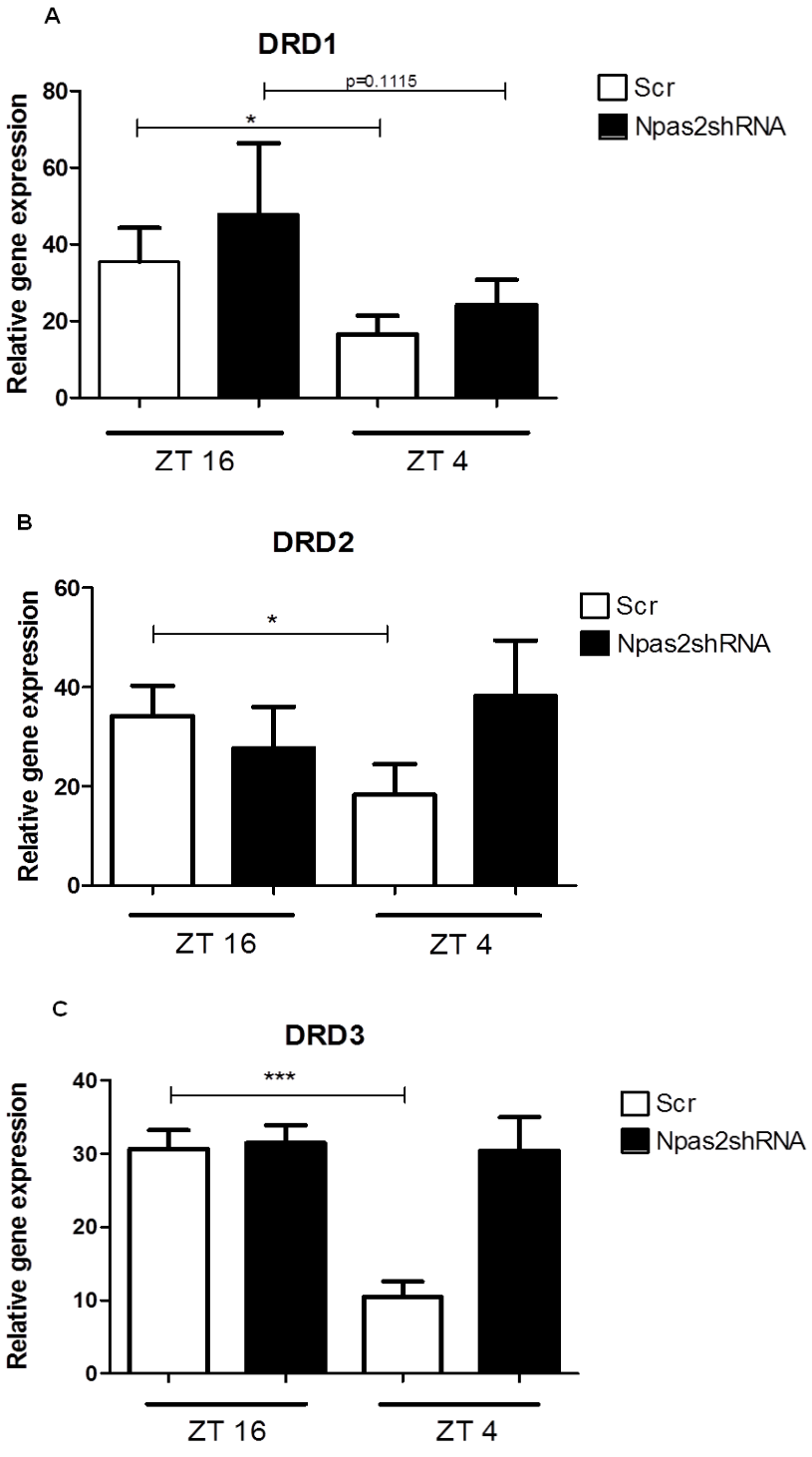
A
DRD1
p=0.1115
*
Scr
Npas2shRNA
Relative gene expression
80
60
40
20
0
ZT 16
ZT 4
B
DRD2
*
Scr
Npas2shRNA
Relative gene expression
60
40
20
0
ZT 16
ZT 4
C
DRD3
***
Scr
Npas2shRNA
Relative gene expression
40
30
20
10
0
ZT 16
ZT 4

Figure 5.2 Effect of *Npas2*k knockdown in diurnal expression of dopaminergic receptors in the NAc. (A) *Npas2* knockdown disrupts diurnal expression of *DRD1*, although a trend towards an increase in expression at ZT 16 is observed (black bars). (B) *DRD2* diurnal expression is abolished following *Npas2* knockdown in the NAc. (C) *Npas2* knockdown abolishes diurnal expression of *DRD3* in the NAc. All AAV-Scrambled mice exhibited rhythmicity, with higher expression at ZT 16 (*$p<0.05$, ***$p<0.001$ by t-test, data is mean ± SEM, n=5-7).

Chronic cocaine disrupts rhythmic expression of dopaminergic receptors in the NAc

Our previous studies (Chapter Three) found that chronic cocaine disrupts rhythmic expression of circadian genes in striatal regions. Thus, the effect of repeated cocaine exposure on rhythmic expression of dopaminergic receptors was assessed in the NAc. Chronic cocaine blunts DRD1 expression rhythms in the NAc ($F_{(5,38)}$=4.068, p=0.0047 saline; $F_{(5,43)}$=1.262, p=0.2978 cocaine, Figure 5.3A). A two-way ANOVA revealed a very significant effect of time ($F_{(5,81)}$=4.15, p=0.0021). An upregulation at ZT4 after cocaine treatment was observed (*p<0.05). Cocaine treatment did not affect rhythmicity of DRD2, save for an upregulation at ZT4 ($F_{(5,38)}$=9.038, p<0.0001 saline; $F_{(5,42)}$=3.263, p=0.0141 cocaine; *p<0.05, Figure 5.3B). A two-way ANOVA revealed a highly significant effect of time ($F_{(5,80)}$=8.52, p<0.0001). An even more striking reduction in DRD3 rhythmic expression was produced by cocaine than DRD1 ($F_{(5,25)}$=6.747, p=0.0004 saline; $F_{(5,26)}$=1.215, p=0.332 cocaine). A two way-ANOVA revealed significant interaction effect ($F_{(5,51)}$=2.83, p=0.0249); a highly significant treatment effect ($F_{(1,51)}$=18.46, p<0.0001); and a highly significant effect of time ($F_{(5,51)}$=7.37, p<0.0001).

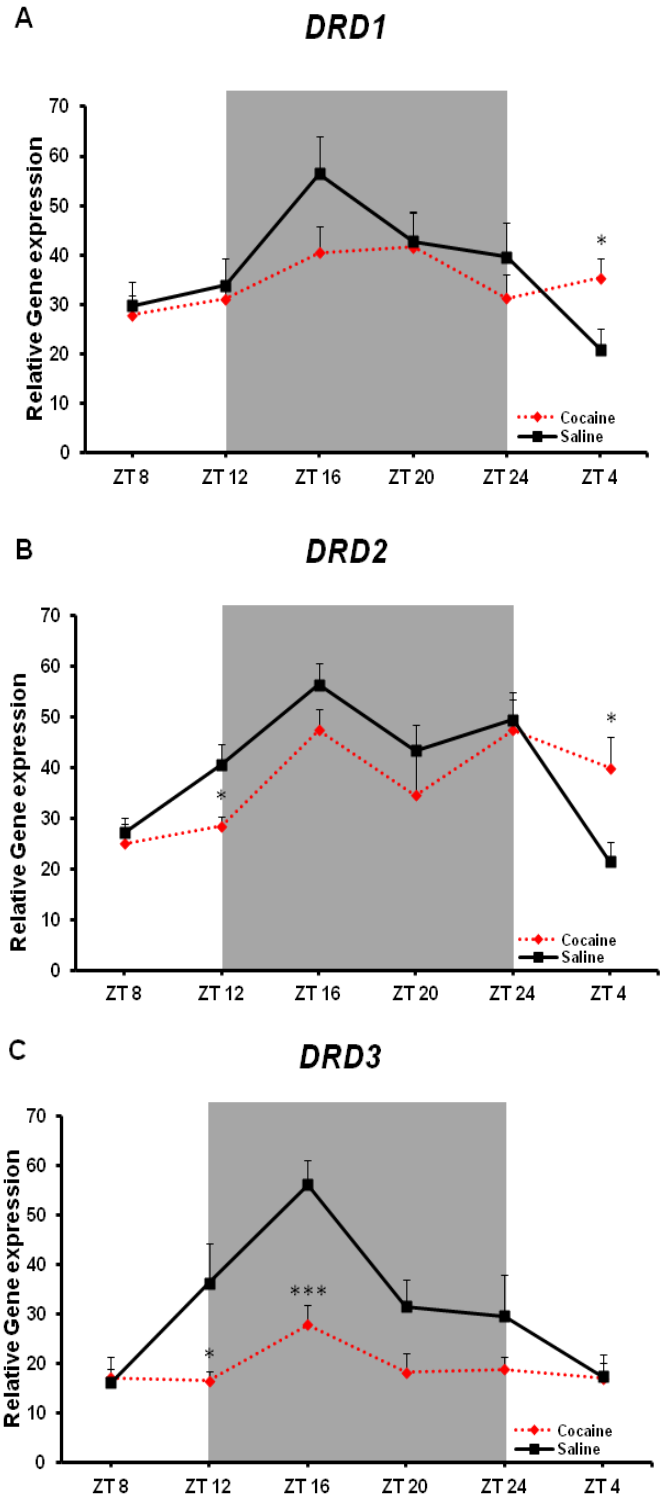
A
DRD1
Relative Gene expression
70
60
50
40
30
20
10
0
ZT 8
ZT 12
ZT 16
ZT 20
ZT 24
ZT 4
*
Cocaine
Saline
B
DRD2
Relative Gene expression
70
60
50
40
30
20
10
0
ZT 8
ZT 12
ZT 16
ZT 20
ZT 24
ZT 4
*
*
Cocaine
Saline
C
DRD3
Relative Gene expression
70
60
50
40
30
20
10
0
ZT 8
ZT 12
ZT 16
ZT 20
ZT 24
ZT 4
*
***
Cocaine
Saline

Figure 5.3 Chronic cocaine alters diurnal expression of *DRD* genes in the NAc. (A) Cocaine alters *DRD1* expression in the NAc ($F_{(5,38)}$=4.068, p=0.0047 saline; $F_{(5,43)}$=1.262, p=0.2978 cocaine). A significant upregulation at ZT4 was observed (*p<0.05). (B) *DRD2* rhythmicity is unaltered in the NAc following cocaine treatment ($F_{(5,38)}$=9.038, p<0.0001 saline; $F_{(5,42)}$=3.263, p=0.0141 cocaine). A significant upregulation at ZT 4was observed (*p<0.05). (C) Cocaine significantly disrupts rhythms in *DRD3* ($F_{(5,25)}$=6.747, p=0.0004 saline; $F_{(5,26)}$=1.215, p=0.332 cocaine). Data is mean ± SEM, n= 5-9.

Discussion

Circadian control of the dopaminergic system seems to be one of the reasons for the time-dependent differential responses to psychostimulants (Manev and Uz, 2009). In fact, many of the components of this system exhibit circadian rhythms in multiple brain regions. Moreover, the dopaminergic system can influence the circadian system as well. Increasing evidence suggests that dopamine may be involved in the regulation of extra-SCN clock gene expression.

Studies have shown that dopamine receptor agonists can induce or repress expression of circadian genes in striatal neurons as well as the retina (Imbesi et al., 2009; Yujnovsky et al., 2006). In fact, an increase in *Npas2* expression has been observed following administration of a D1, and not D2, agonist, whereas *Clock* expression was both increased and decreased following administration of D1 and D2 agonists, respectively (Imbesi et al., 2009). These studies start to suggest that effects on the circadian machinery can be differentially mediated by the different dopaminergic receptors. In this study, NPAS2 was found to bind only DRD1 and DRD3 promoter regions, whereas CLOCK was able to bind all three receptors' promoters. It is intriguing to speculate that NPAS2 could be mediating effects through or colocalizing with D1 expressing neurons, whereas CLOCK can promote effects via D2 expressing neurons. In fact, a study performed in retinal cells found that D2-mediated signaling potentiated the transcriptional capacity of CLOCK:BMAL1 complexes (Yujnovsky et al., 2006). Even though the D2-like D3 receptors were the most regulated by Npas2, these have been found to be expressed in D1-expressing

neurons in the NAc, further supporting the idea that Npas2 might act through this D1 neuronal population(Surmeier et al., 1996). Additionally, this study showed that NPAS2 is necessary for proper maintenance of rhythmic expression of dopamine receptors, presumably via direct mechanisms in the case of DRD1 and DRD3 receptors, and indirectly in the case of DRD2, since no binding by NPAS2 was observed at this promoter.

Interestingly, chronic cocaine disrupts rhythmic expression of DRD1 and DRD3 in the NAc, similar to that observed in *Npas2* (Chapter Three). Thus, the flattening or blunting observed in Npas2 can lead to or is a consequence of disrupted dopamine receptor expression. *DRD2* rhythmic expression was not changed in the NAc, similar to that observed in *mPer2* in the NAc. Studies have shown that activation of D2 receptors by endogenous dopamine regulates the expression of *rPer2* (Hood et al., 2010). Thus, it seems that cocaine might regulate the expression of NPAS2 and CLOCK via D1 and D2 receptor signaling, respectively, and these, in turn, can regulate rhythmic expression of dopaminergic receptors. These disruptions by cocaine highlight the presumed Zeitgeber ability of the drug, since they can be interpreted as the effect of a clash between the drug and light's synchronizing influences. Further studies are required to investigate the exact molecular mechanism of this core-circadian clock regulation of dopamine receptors. Npas2 is emerging as an important regulator of the dopaminergic system in the striatum, much like Clock seems to be in midbrain regions. Not only does Npas2 is involved in dopamine receptor expression, but it has been shown to be a direct regulator of MAOA in the

striatum, one of the enzymes involved in degrading dopamine. Further studies should look at the state of the dopaminergic system in the Npas2 mutant mice. The ClockΔ19 mouse displays altered dopaminergic neurotransmission, which seems to underlie its mood or anxiety-related abnormalities. Thus, it would be interesting to study other circadian mutants and further support the connection between disturbed clock function and altered dopaminergic transmission or dopamine-related behaviors.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

Drug addiction as well as other psychiatric disorders has become increasingly associated with the regulation and/or dysfunction of circadian rhythms (Menet and Rosbash, 2011). Many drug addicts as well as patients that suffer from mood disorders exhibit behavioral and physiological abnormalities related to a dysfunctional circadian clock. These disruptions in circadian-related behaviors are thought to be mediated by a dysregulation of circadian control of neurotransmitter systems, like dopamine and serotonin. However, the interaction between the circadian and reward systems is bidirectional (Webb et al., 2009). Cocaine is known to affect a variety of circadian-associated events and on the other hand, the circadian system regulates responses and timing of cocaine effects. This thesis aimed to further understand the effects that cocaine has on the circadian machinery in striatal regions as well as the role that circadian genes might play in the regulation of cocaine reward. Following chronic cocaine treatment, expression of the *Per* genes was found to be differentially induced in the NAc and CP. Regulation of these core-circadian clock genes were mediated through NPAS2, a protein similar to CLOCK in structure and function. NPAS2 has been found to be able to compensate for loss of CLOCK in the mammalian forebrain (Reick et al., 2001). Expression of NPAS2 is the reason why *Clock* knockout mice display no abnormalities in locomotor activity rhythms (Debruyne

et al., 2006). Additionally, rhythmic expression of circadian genes was disrupted by chronic cocaine in both the NAc and CP. In fact, a disruption in *Npas2* rhythms was observed in the NAc, whereas no effect was observed in *Clock* expression. Interestingly, a significant upregulation was observed at a timepoint approximately 48 hrs after the last drug injection and two hours prior to subjective drug administration. This suggests a persisting effect in circadian gene expression that could modulate anticipatory behavior observed after chronic administration of drugs of abuse. Similar to food, most drugs of abuse (alcohol, methamphetamine, cocaine, nicotine and opioids) display the ability to entrain behavioral locomotor activity rhythms (White et al., 2000; Kosobud et al., 2007; Gillman et al., 2008; Gillman et al., 2009; Shibata et al., 2010). The idea that drugs of abuse could entrain behavior led to the concept of extra-SCN drug-entrainable oscillators in specific brain regions, which some studies have suggested to reside in the mesolimbic dopaminergic system, a common molecular pathway implicated in responses to drugs of abuse (Ijima et al., 2002; Nestler et al., 2005). These would function in a similar way as the food-entrainable oscillator, which controls anticipatory behavior to the presentation of food (Rossenwasser et al., 1981). Indeed, studies have shown that these two oscillators are two different systems that can be activated by metabolic stimuli or reward. Both normal food- and chocolate-entrainment do not disrupt diurnal expression of *Per1* in the SCN, but each one have differential effects in other brain regions (Angeles -Castellanos, et al., 2008). Food entrainment only affects *Per1* expression in hypothalamic regions, whereas chocolate entrainment affects

Per1 levels in other regions like the NAc, amygdala and PFC, regions involved in regulating responses to drugs of abuse and addictive behaviors (Angeles-Castellanos, 2008; Koob and Volkow, 2009). The fact that cocaine does not alter circadian gene or protein expression in the SCN does not exclude it from influencing activity in the SCN. In fact, a recent study found that even a single acute systemic injection of cocaine can alter neuronal activity in the SCN, observed as phase shifts in the circadian firing of SCN neurons (Glass et al., 2012). How the SCN can communicate with the midbrain dopaminergic system in order to affect its activity is still not clearly understood. A study by Luo and Jones found a novel projection from the SCN to the VTA via the medial preoptic nucleus (MPON). This circuit may be a substrate for the circadian regulation of reward, but the clock genes are expressed throughout the brain, thus involving many other brain regions in this regulation. The ability of drugs of abuse to act as synchronizers could open the door to possible chronopharmacological therapies in addiction, like administration of methadone or buprenorphine at specific times of day to maximize their effects. Further studies are needed to deepen our understanding on the localization and regulation of these oscillators by other systems, i.e. hormones and peptides. It is important to note that the studies performed in this thesis use a non-contingent delivery of drug. Characterization of circadian gene disruptions should be performed in mice that self-administer cocaine for several weeks, but a great number of mice would be needed for the different timepoints throughout the day.

To further the understanding of the circadian genes in drug reward, especially cocaine, circadian mutant lines were first tested for their preference for cocaine via conditioned place preference. Interestingly, the *Npas2* mutant mice displayed a preference phenotype opposite of that observed in the *Clock* Δ19 mutant mice. Both NPAS2 and CLOCK are transcription factors, so this discrepancy in cocaine preference is presumed to be as a result of their effect on gene regulation. In fact, it is possible that a differential modulation of dopaminergic activity could be at the root of this phenotype contrast. Clock has been recently found to be a negative regulator of TH in the VTA, whereas Npas2 can regulate dopaminergic receptor expression in the NAc (Spencer et al., submitted; and present study). Indeed, Clock mutant mice show an enhanced dopaminergic activity that might be the cause of their behavioral abnormalities (Spencer et al. submitted). Future studies should examine the direct targets of these two proteins following saline and cocaine treatment. Not only will this help clarify their role in regulating drug preference but it will probably reveal new players directly regulated by the circadian system that could be potential targets for chronotherapeutics of neuropsychiatric disorders. In order to identify the region where these genes might be involved in regulating drug reward, viral-mediated knockdown was performed in the NAc, a region involved in motivated behavior and part of the dopaminergic system (Di Chiara et al., 2002). Knockdown of *Npas2* in the NAc led to a decrease in cocaine preference, similar to the mutant mice, suggesting a role for NPAS2 in the NAc in the regulation of drug reward. Furthermore, *Clock* knockdown in this area resulted in no effect on

cocaine preference; perhaps a not so surprising result since CLOCK has been shown to mediate mood-associated behaviors and dopaminergic neurotransmission in the VTA (Mukherjee et al., 2010). Microarray studies were performed on NAc tissue from *Npas2* mutant and wt mice. These studies demonstrated that a significant number of the genes regulated by an absence of NPAS2 were similar to those observed in the NAc of mice that over-expressed CREB (McClung and Nestler, 2003). The *Npas2* mutant mice exhibited an increase in levels of P-CREB in the NAc, which could help explain both the increase in CREB regulated genes and the decrease in cocaine preference as measured by CPP (unpublished observations). Mice that overexpress CREB in the NAc were found to display a decrease in reward via CPP (Carlezon et al., 1998). It will be important in future studies to understand how a mutation in NPAS2 leads to changes in P-CREB, and whether these changes underlie the behavioral phenotypes of the *Npas2* mutant mice.

Per mutant mice displayed an increase in cocaine preference in single *mPer1* and *mPer2* lines. The double mutant mice showed a synergistic effect in the behavioral preference for cocaine. When these genes are knocked-down in the NAc, a strong trend towards an increase in cocaine preference was observed. Future studies could investigate whether the Per double mutant mice have altered dopaminergic neurotransmission, focusing on dopamine receptor expression and function in the striatum. Interestingly, the double *mPer1;mPer2* mice also show an increase in behavioral measures of anxiety (See Appendix). This anxiety phenotype was also observed in mice with a knockdown of both

genes in the NAc, suggesting a role for the Per proteins in the NAc in regulating anxiety-like behaviors. The double Per mutant mouse was found to exhibit a decrease in PCREB in the NAc (unpublished observations), which could help explain the increase in preference observed by CPP and the increase in anxiety. Studies that manipulate CREB via viral-mediated gene transfer indicate that disruption of CREB function in the NAc increases reward to cocaine (as measured by CPP) and increases anxiety-like behaviors, similar to that observed in the *mPer1;mPer2* mutant mice (Carlezon et al., 1998; Barrot et al., 2002; Barrot et al., 2005). These studies should be extended to the more clinically relevant model of addiction, the self-administration paradigm, since differences have been observed in terms of CREB's role in reward when studied using a passive versus volitional drug administration (Larson et al., 2011). It is possible that these mutations or knockdowns may lead to different behavioral outcomes in self-administration paradigms. Future studies should also focus on the role of these circadian genes in other brain regions involved in mood and reward. Now that a tool to study the role of these specific circadian genes is available, the studies would be straightforward. Additionally, since *mPer3* was highly regulated following cocaine in both striatal regions, further studies should be performed in order to study the role of this gene in reward and mood-related behaviors. In fact, variants of the human *Per3* gene have been associated in a number of mood disorder features, like age of onset, response to SSRIs, mood oscillations and characteristics of temperament, further highlighting the role of this gene in

psychiatric disorders since it has little effect in basic circadian function in the SCN (Artioli et al., 2007).

Another finding of these studies is that CLOCK and NPAS2 directly bind to the promoter regions of dopaminergic receptors, suggesting a potential direct circadian control in their expression. Interestingly, NPAS2 was only able to bind *DRD1* and *DRD3* promoters, whereas CLOCK was able to bind all of three of them. When *Npas2* was knocked-down specifically in the NAc, the diurnal expression of all three receptors was abolished. How *DRD2* diurnal variation was abolished is unclear, as NPAS2 was not found to bind its promoter. It is likely that a change in expression of the D3 receptor could result in compensatory changes in D2 receptor expression, thus leading to an indirect effect on expression. Because dopaminergic receptors show a diurnal variation in the NAc, the effect of chronic cocaine on this rhythmicity was also measured. Interestingly, chronic cocaine was able to disrupt diurnal variation of *DRD1* and *DRD3*, but not *DRD2.* The effect on DRD1 and DRD3 is similar to that observed for Npas2 following chronic cocaine in the NAc (Chapter 3), and being that Npas2 can bind to the promoter of these two receptors and regulate their expression, it is tempting to speculate that the rhythmic disruption observed in the dopamine receptors could be an effect of a rhythmic disruption in Npas2 expression and function. Moreover, DRD2 rhythmic expression in the NAc was not disrupted following chronic cocaine, and again, Npas2 was not able to bind to this receptor's promoter. Previous studies have looked at how dopamine receptor activation regulates circadian gene expression in striatal neurons and found that *Npas2* expression is

upregulated following a D1, but not D2, agonist treatment (Imbesi et al., 2009). *Clock* expression was also found to be upregulated by a D1 agonist, while a D2 agonist leads to a decrease in its expression, but does not affect *Npas2* expression (Imbesi et al., 2009). However, in other regions like the retina, D2 receptor activation can potentiate Clock function (Yujnovsky et al., 2006). Additionally, Clock was found to colocalize with D2-expressing striatal neurons (Imbesi et al., 2009). All these findings suggest a possible role for these two transcription factors in the two different populations of neurons in the striatum, D1-expressing versus D2-expressing neurons. However, this is not clear cut since these populations are not completely exclusive, and neurons can express both D1 and D2 receptors, as these can form heterodimers with each other or in combination with D3 or D5 receptors (Surmeier et al., 1996; Maggio et al., 2009). If these proteins are not distinctly colocalized with a particular type of striatal neuron, then their effects will strictly depend on them having different molecular targets or opposing effects on the same targets, which is why ChIP-sequencing studies are necessary to discover these targets in the striatum. Future studies should also investigate the functional consequences of these cocaine-induced disruptions in rhythm, possibly by studying DA receptor signaling across the day following chronic cocaine or saline treatments. In addition, further exploration of D3 receptor rhythmic function or disruption is warranted.

Overall, the findings from this study provided more evidence for the striatum as a site of a drug-entrainable oscillator of molecular rhythms. Additionally, combining published data with results from this study led to the

development of a speculative model where Npas2 and the Per genes regulate reward and anxiety in the NAc in different ways, as well as modulate the rhythmic expression of different dopaminergic receptors in the ventral striatum (Fig 6.1). In the NAc, cocaine can activate NPAS2, presumably via D1R signaling, which can drive expression of *Per* genes and regulate dopamine receptor expression. Given that *Npas2* mutant mice exhibit an increase in P-CREB, then under basal conditions NPAS2, part of a circadian loop with the PER proteins, could be inhibiting CREB functions. When NPAS2 is not present, this inhibition is lost and CREB activity rises, which leads to reduced drug reward as measured by CPP. When the PER proteins are not present, inhibition of NPAS2 is lost, which leads to a reduction in CREB activity, and thus increased cocaine preference and anxiety-like behaviors. Additionally, NPAS2 can modulate rhythmic expression of D1R and D3R. A disruption in this rhythmicity, via Npas2 reduction/deletion or chronic cocaine could result in altered dopaminergic neurotransmission. Presumably, from our current data, a blunting or disruption of this rhythm could maintain dopamine receptor levels constant throughout the day, and even in lower levels than normal, possibly resulting in decreased receptor functioning and cocaine-mediated behavioral response, like the one observed in the Npas2 mutant mice. Maintaining proper rhythmicity of the circadian machinery is integral to our physiology and health, since dysfunction in circadian timing systems have been associated with a number of diseases, from neuropsychiatric disorders like addiction to even cancer (reviewed in Yu and Weaver, 2011). Indeed, studies have shown that mice with a circadian misalignment/disruption, caused by

chronic exposure to a 10:10 Light/Dark cycle, show an impulsive-like phenotype (Karatsoreos et al., 2011). Impulsivity is one of the most studied predisposing factors to the development of addiction, where many animal and human studies suggest that it precedes drug-taking behaviors (reviewed by Dalley et al., 2011). Thus, circadian dysfunction might increase vulnerability to drug addiction not just by its impact on mood, anxiety and sleep, but also by its impact on cognitive control.

The blunting of rhythmic expression in striatal regions by chronic cocaine might also lead to disruptions in mesolimbic circuit activity. ClockΔ19 mice, which have severely disrupted circadian rhythms and an increased reward-value for cocaine, have been shown to have deficits in NAc phase signaling (Dzirasa et al., 2010). Neural phase signaling is considered to be essential in cognitive processing. Several studies have implicated phase signaling as a coding mechanism through which the brain combines the activity of neurons across different brain areas to produce thoughts and behaviors (Lisman and Buzsaki, 2008). Specifically, ClockΔ19 mice show a dysfunction in low gamma phase coupling specifically in the NAc, which suggests an alteration in the activity of NAc microcircuits (Dzirasa et al., 2010). Cross-functional phase coupling (CFPC) is a phenomenon where high-frequency neural oscillatory activity is modulated by low-frequency modulatory activity. CFPC has been found to be important in coordinating neural oscillatory activity during high end cognitive processes, as well as being one of several aspects important to neural phase signaling (Palva et al., 2005). Interestingly, wild type mice that underwent a

sensitization regime exhibited similar also display a disruption in NAc CFPC, similar to that observed in the ClockΔ19 mice (unpublished observations).However, it is unclear whether this is a result of chronic cocaine-induced circadian disruption or if it is a parallel effect of chronic drug use alongside the effects on rhythmic gene expression. Thus, disruption of rhythms by chronic cocaine might promote addictive behaviors by altering dopamine receptor functioning or causing deficits in specific microcircuit functioning which can lead to changes in cognitive performance. Studying the interactions between the circadian and reward system is important since these systems are in continuous interplay in regulating the timing and behavior towards natural rewards, like food and sex, that are important for a species survival, as well as other rewards, like drugs of abuse.

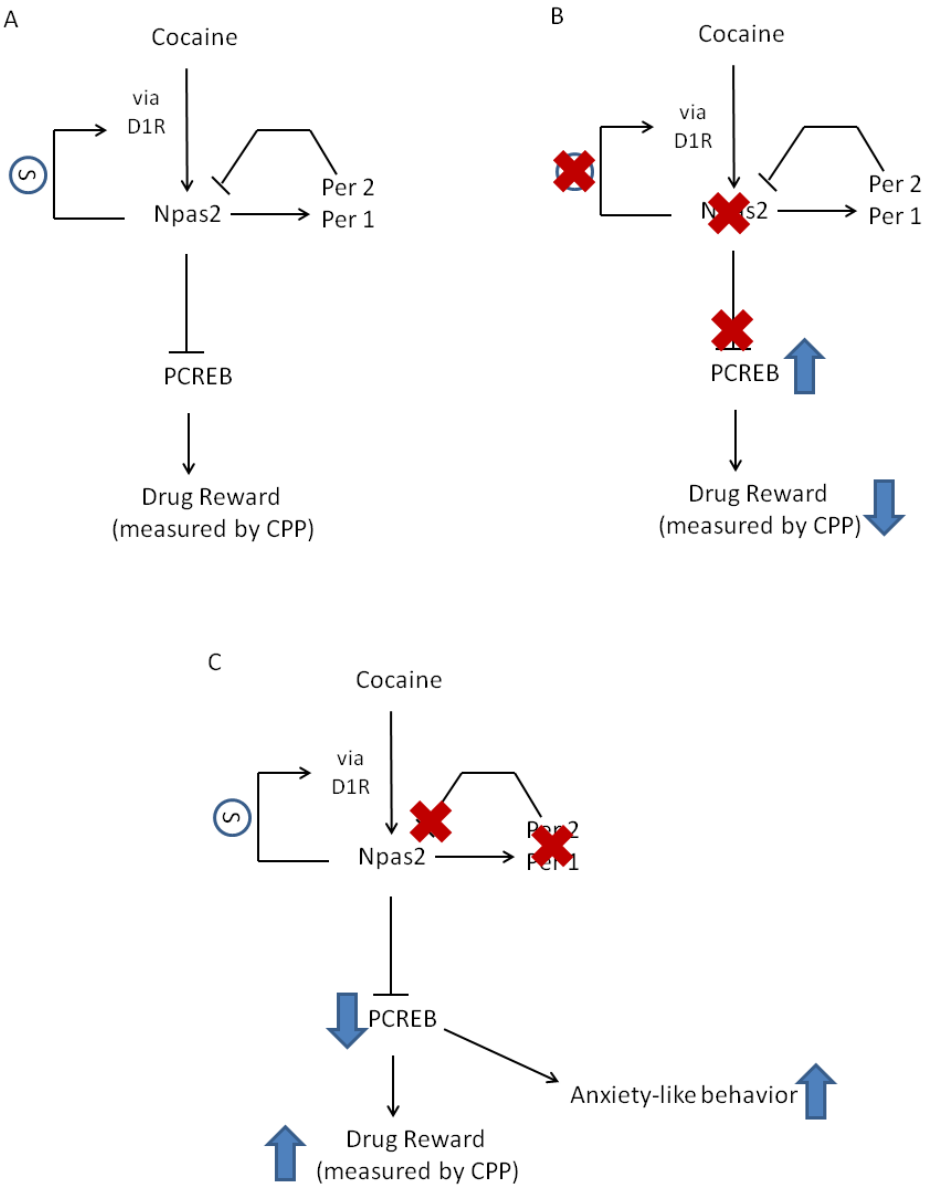

Figure 6.1 Speculative Model of interactions between circadian genes, DA Receptors, PCREB and behavioral outcomes. (A) Wild-type scenario. (B) Npas2KO mice. (C) mPer1/mPer2 mutant mice. The circle with an inverted S indicates rhythmic regulation. See text for further explanation.

APPENDIX

CIRCADIAN GENES *PERIOD 1* AND *PERIOD 2* IN THE NUCLEUS ACCUMBENS REGULATE ANXIETY-RELATED BEHAVIOR

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Introduction

Circadian rhythms are prominent in virtually every species on this planet and nearly all bodily and cognitive functions in humans follow a 24 hr cycle (Ko and Takahashi, 2006). Several studies have found that disruptions in normal rhythms in the sleep/wake cycle can lead to a variety of health problems such as jet lag, shift workers syndrome, and even increase the risk for cancer and heart disease (Moser et al., 2006). It has become increasingly clear that circadian rhythms also contribute to differences in mood state, reward and motivation. Abnormal rhythms are strongly associated with psychiatric diseases like seasonal affective disorder (SAD), bipolar disorder, major depression, and drug addiction (Falcon and McClung, 2008; McClung, 2007). Moreover, many of the treatments used for these illnesses are known to modulate the circadian clock. Nonetheless, the exact role of rhythm disruptions in these diseases still remains

elusive, as does the contribution of specific circadian genes in individual brain regions in the regulation of mood.

The circadian clock in mammals is regulated by a core transcriptional/ translational loop which cycles over the course of 24 hrs (Ko and Takahashi, 2006). The suprachiasmatic nucleus (SCN) is the location of the central pacemaker in the brain, however most circadian genes are widely expressed throughout the brain and in other organs. The central components are the Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-like Protein 1 (BMAL1) proteins which dimerize and induce the expression of the *Period* (*Per1, Per2,* and *Per3*) and the *Cryptochrome* (*Cry1* and *Cry2*) genes. The CRY and PER proteins can inhibit the activity of CLOCK and BMAL1, thus creating a negative feedback loop. Several other proteins such as Rev-erbα, Retinoid-Related Orphan Receptor Beta (RORβ), Casein kinase 1 epsilon (CK1ε), Glycogen Synthase Kinase 3 Beta (GSK3β), are also involved in regulating the timing of these rhythms. It was shown previously that the three *Period* genes have differential functions in the regulation of circadian rhythms (Bae et al., 2001; Shearman et al., 2000). Mice with a mutation in either *mPer1* or *mPer2* have disrupted free running rhythms with lower amplitude in constant darkness (DD). However, most mice retain a significant period of approximately 22-23 hours for several days in DD before losing all rhythmicity (Bae et al., 2001). Animals with mutations in both *mPer1* and *mPer2* have a much more dramatic and immediate loss of rhythmicity in DD, suggesting that PER1 and PER2 can compensate for one another to some extent when one protein is lost

to help maintain circadian rhythms (Bae et al., 2001). In contrast, mice with a mutation in *mPer3* have surprisingly few disruptions in circadian locomotor activity. Indeed, mice with double mutations in *mPer3* and *mPer1* or *mPer2* do not have an increase in circadian rhythm disruption over the single *mPer1* or *mPer2* mutations alone, suggesting that this gene has a minimal role in core circadian clock function even though it displays rhythmic expression levels in the SCN (Bae et al., 2001; Shearman et al., 2000). However, recent studies suggest a more tissue-specific role for *mPer3* in circadian timekeeping of peripheral oscillators throughout the body, like the liver, lung, and esophagus, among others (Pendergast et al., 2012)

Several human genetic studies have identified SNPs and haplotypes in individual circadian genes that associate with various psychiatric disorders. For example, variations in *Clock*, *Bmal1*, *GSK3β*, *Per3*, *RORβ* and *Rev-erbα* have all been linked to a bipolar disorder diagnosis or various aspects of bipolar disorder (Benedetti et al., 2004; Benedetti et al., 2007; Kishi et al., 2008; Kripke et al., 2009; McGrath et al., 2009; Nievergelt et al., 2006). Moreover, polymorphisms in *Per2* are associated with vulnerability to major depression and seasonal depression (Lavebratt et al., 2010; Partonen et al., 2007) and levels of *Clock*, *Period 1* and *Bmal1* mRNA are elevated in blood leukocytes of people with a history of depression (Gouin et al., 2010). Recently, SNPs in *Bmal2* (a functional homologue to *Bmal1*) have been identified that associate with social phobia anxiety-related disorders (Sipila, 2010). Work in animal models has found that mice with a mutation in the *Clock* gene (*Clock*Δ19) (King et al., 1997; Vitaterna et

al., 2006) have a complete behavioral profile which is strikingly similar to human bipolar patients in the manic state, including hyperactivity, lowered levels of anxiety or increased “risk taking” behavior, lowered levels of depression-like behavior, and an increase in the reward value for a variety of stimuli (McClung et al., 2005; Roybal et al., 2007). A few studies have similarly begun to examine the influence of the Per genes on mood and reward related behavior in mice. (Abarca et al., 2002; Halbout et al., 2011; Spanagel et al., 2005). Here we wanted to examine the influence of the *Per* genes on anxiety-related behavior and start to understand the mechanisms by which these genes are involved in the response to stress.

Materials and Methods

Mice

Homozygous *mPer1*^{ldc}, *mPer2*^{ldc}, and *mPer1;mPer2*^{ldc} mice used in this study were generously provided by David Weaver and colleagues at UMass Medical School (Bae et al., 2001; Shearman et al., 2000) and bred and genotyped at UT Southwestern Medical Center at Dallas. All mutant mice were on a 129sv background. Individual wild type (WT) littermates generated from heterozygote breeding were used as controls for all single gene knock-outs and combinations of these WT animals from the single mutation crosses were compared to the double knock-out animals (which were maintained as homozygotes for both mutations). No behavioral differences were found between WT animals from the *mPer1* or *mPer2* crosses (data not shown). Mice

used in social defeat experiments are described in detail below. All animals were maintained in a 12:12 light/dark cycle (lights on at 7am) with food and water freely available. Experimental mice used in behavioral analysis were adult males (8-12 weeks old) and behavioral tests of locomotor activity, anxiety, and depression-related behavior were conducted between ZT 3-6 (social defeat experiments are detailed below). All procedures were approved by our Institutional Animal Care and Use Committee at UT Southwestern.

Behavioral Tests

Locomotor response to novelty: Mice were individually placed in automated locomotor activity chambers equipped with infrared photobeams (San Diego Instruments, San Diego, CA) and measurements began immediately. Activity of the animal was continuously measured and the data was collected in 5-min blocks over a period of 2 hours.

Elevated Plus Maze: Mice were placed in the center of an elevated plus maze (arms are 30×5 cm, with 25 cm tall walls on the closed arms) under low light levels and their behavior was monitored for 5 min. The time spent on the closed and open arms, as well as the number of explorations of open and closed arms were determined by video tracking software, Ethovision 3.0 (Noldus, Leesburg, Virginia). Time spent on the open arm and percent of entries into the open arm are both negatively correlated with anxiety-like behavior. The apparatus was cleaned and allowed to dry between every mouse.

Dark/Light Test: The dark/light apparatus consisted of 2-chambered boxes (25 cm x 26 cm for each side, Med Associates, St. Albans, Vermont). One side was kept dark (room light entry limited) and the other side was brightly lit by a fluorescent bulb across the top. Mice were first placed in dark side for 2 min, then the door between the compartments was opened and they were allowed to freely explore either the light or dark side for 10 min. Anxiety-like behavior was measured as the time spent in the lit side during the final 10 min.

Open field test: Mice were placed in the periphery of a novel open field environment (44 cm x 44 cm, walls 30 cm high) in a dimly lit room and allowed to explore for 5 min. The animals were monitored from above by a video camera connected to a computer running video tracking software (Ethovision 3.0, Noldus, Leesburg, Virginia) to determine the time, distance moved and number of entries into two areas: the periphery (5 cm from the walls) and the center (14 cm x 14cm). The open field arenas were wiped and allowed to dry between mice.

Social Defeat and Avoidance Testing

Social defeat and avoidance testing were performed according to published protocols (Berton et al., 2006; Krishnan et al., 2007; Tsankova et al., 2006). Briefly, CD1 retired breeder mice were screened for consistent attack latencies (<30 sec on 3 consecutive screening sessions with a C57Bl/6ByJ intruder). C57Bl/6ByJ were purchased from Jackson laboratory and group housed in a 12 hr light/dark cycle with food and water *ad libitum* for at least one week prior to the defeat protocol in our facility. During each defeat episode,

intruder mice were allowed to interact for 10 min with the aggressive CD1 mouse, during which they were attacked and displayed subordinate posturing. Non-defeated controls were housed in identical cages opposite each other and were rotated similarly. Immediately after the tenth defeat all mice were singly housed. Social defeat was always performed in the few hrs before the onset of the dark phase (1730-1830 hrs). Social interaction tests were performed 24 hrs after the last defeat and then again after the last antidepressant treatment (imipramine 20 mg/kg i.p. daily for 28 days). On these days the time spent in the interaction zone during the first (target absent) and second (target present) trials were measured and the *interaction ratio* was calculated as 100 × (interaction time, target present)/(interaction time, target absent). Previous work has found that depression-related behavior persists 28 days after the social defeat protocol and that imipramine treatment completely reverses the social interaction deficit seen in susceptible animals (Berton et al., 2006; Tsankova et al., 2006). Animals were killed 24 hours after the last imipramine or vehicle treatment and NAc dissections were taken by punch dissection as described previously (McClung and Nestler, 2003).

Quantitative PCR

cDNA was mixed with buffer, primers, SYBR green, and hot start Taq polymerase in a prepared master mix (Applied Biosystems). PCR reactions followed by a dissociation reaction to determine specificity of the amplified product were run on a Real-Time PCR machine (7300 Real Time PCR machine,

Applied Biosystems). The amount of gene expression was quantified using the ΔΔCt method as previously described (LaPlant et al., 2009). The following primer sets were used to measure *Per* expression: mPer1 For – CT CTGTGCTGAAGCAAGACCG; mPer1 Rev – TCATCAGAGTGGCCAGGATCTT; mPer2 For – GAGTGTGTGCAGCGGCTTAG; mPer2 Rev – GTAGGGTGTCATGCGG AAGG.

Microarray analysis

Microarray analysis was performed as described previously (Berton et al., 2006; Wallace et al., 2009). Briefly Affymetrix mouse 430_2 whole genome arrays were utilized with NAc tissue taken from individual *mPer1;mPer2* double mutants (n=3) or WT controls (n=3). Array results were normalized in Array Assist (Agilent Technologies; Stratagene) using the PLIER algorithm. Comparative analysis was also performed in Array Assist utilizing an upaired t-test to compare each experimental group with control animals that were handled, treated, and dissected at the same time. Data files from Array Assist were exported into Excel (Microsoft) and then imported into Genespring (Silicon Genetics) for additional analysis and data visualization. In each condition, genes were considered to be regulated if the raw signal was significant to background, the fold change was greater than 1.36, and the comparison p-value was < 0.01. Pathway analysis was performed using the Gene Set Enrichment Analysis (GSEA; Broad Institute) which utilizes multiple databases including KEGG,

Biocarta, Reactome, and GO. Statistically significant gene sets are determined by Fisher Exact Test ($P<0.05$).

Construction of mPer1;mPer2 shRNA

A small hairpin RNA (shRNA) was constructed against *mPer1* and *mPer2* mRNA by selecting a conserved 24 base sequence (5'-ATCCCTCCTGACAAGAGGATCTTC-3') in the coding region. For the scrambled shRNA, a random sequence of 24 bases (5'-CGGAATTTAGTTACG GGGATCCAC-3') that had no sequence similarities with any known genes/mRNA was used. An antisense sequence of the selected mRNA region followed by a miR23 loop of 10 nucleotide (CTTCCTGTCA) was added at the 5'end of the above sequences. These shRNAs were designed as synthetic duplexes with overhang ends identical to those created by Sap I and Xba I restriction enzyme digestion. The annealed oligonucleotides were cloned into the adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (Stratagene, La Jolla, CA). Plasmids were sent to the University of North Carolina Viral Vector Core for production (Chapel Hill, NC).

Stereotaxic surgery

Stereotaxic surgery was performed similarly to Mukherjee et al 2010. Mice were anesthetized with a mixture of ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline (0.9% NaCl). Bilateral stereotaxic injections of 1 µl of purified high titer AAV encoding scrambled or AAV-

mPer1;mPer2 shRNA was injected into the NAc (from bregma: angle 10°, AP +1.5 mm, Lat +1.5, DV -4.4) using a 33 gauge hamilton syringe (Hamilton, Reno, NV). Injection speed was 0.1 µl/minute, and the needle was kept in place for an additional 5 minutes before it was slowly withdrawn. Mice recovered for two weeks in their home cage prior to behavioral testing to allow for full virus expression.

Immunohistochemical localization of AAV expression

Mice were anesthetized with 50 mg/kg Nembutal in saline, and transcardially perfused with 4% paraformaldehyde in 1X PBS (1 mM KH2PO4, 10 M Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The brains were allowed to post-fix in a 4% paraformaldehyde for 24 hours and then placed in 1X PBS-30% glycerol sucrose protection for an additional 24 hours before being stored in 1X PBS-0.05% sodium azide. 30 µm brain sections were obtained with a microtome (Leica, Wetzlar, Germany) and immunohistochemical staining against GFP (AbCam, Cambridge, MA) was carried out using standard procedures . Secondary antibodies (anti-rabbit conjugated with Alexa 488) was purchased from Molecular Probes (Carlsbad, CA). Brain sections were mounted using Vectashield (Vector Labs, Burlingame, CA) with DAPI counterstaining and observed with an epifluorescence microscope with a 10x objective. Animals were excluded from our study if their infection spread was not localized to the NAc , with spillover to adjacent areas or throughout the injection tract; or if there was a significant disproportionate amount of infection between both

hemispheres. Exclusion by these criteria accounted for approximately 10% of animals.

Statistical analysis:

All data are expressed as mean ± standard error of the mean. Significance for two group comparisons in behavioral assays and qPCR analysis was determined by two way ANOVA and post-hoc analysis. Behavioral results from the shRNA experiments were analyzed by student's t-test. In all experiments $P<0.05$ is considered significant.

Results

mPer1 and mPer2 are involved in regulating anxiety-related behavior

To determine if mice deficient in *mPer1*, *mPer2*, or both have any anxiety-related behavioral abnormalities, we utilized three behavioral paradigms, the dark/light test, open field and the elevated plus maze. Each of these tests have been validated extensively as a measure of anxiety-related behavior and for their sensitivity to anxiolytic and anxiogenic drugs (Belzung and Griebel, 2001). Compared to WT animals, we found a significant decrease in the activity on the light side of the dark/light chamber in mice deficient in *mPer1* and a more sizeable decrease in activity in mice lacking *mPer1*and *mPer2* (Figure A.1A). Mice deficient in *mPer2* alone were similar to WT animals.

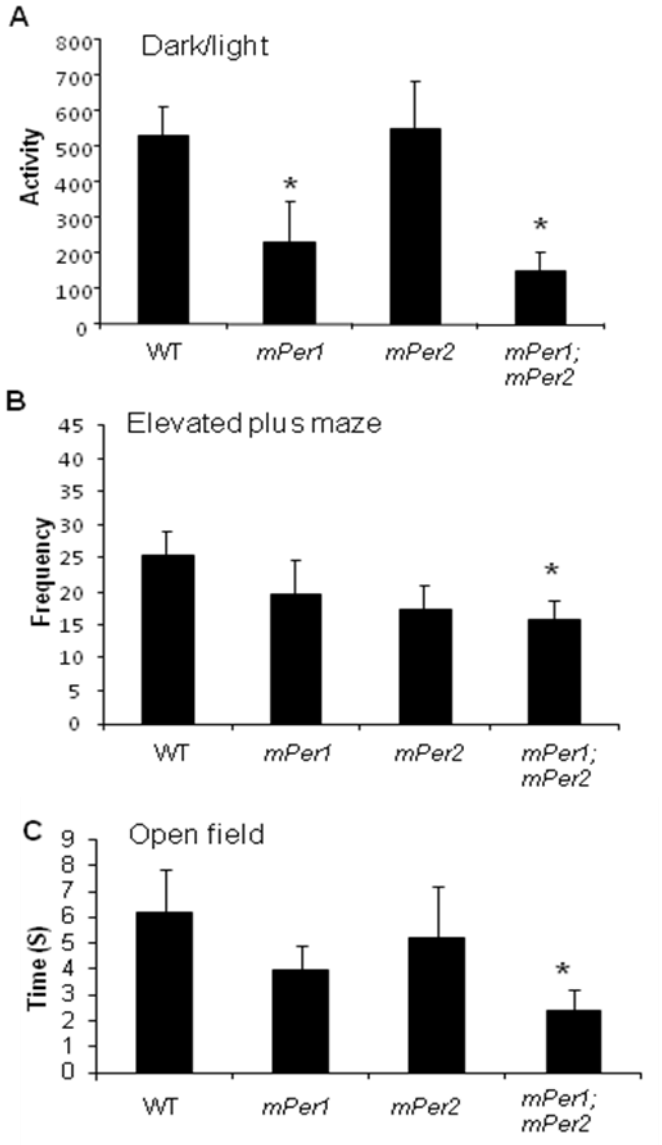

Figure A.1 *mPer1;mPer2* mutant mice are more anxious. Mice were subjected to the multiple measures of anxiety-related behavior. *mPer1* mutant mice and *mPer1;mPer2* mutant mice had less activity in the lit compartment of a dark/light box compared to WT mice (A). *mPer1;mPer2* mutant mice had less frequent entries into the open arm of the EPM (B). *mPer1;mPer2* mutant mice spent less time in the center of the OF (C; n=12-24, *P<0.05).

In the elevated plus maze there was a significant decrease in the frequency of open arm entries in *mPer1;mPer2* mutant mice with nonsignificant reductions in each single mutant alone (figure A.1B). In the open field test, *mPer1;mPer2* mutant mice spent less time in the center compared with WT mice (Figure A.1C). Again animals deficient in only *mPer1* or *mPer2* were not significantly different than WT. Taken together, these results suggest that *mPer1;mPer2* mutant mice have an increase in anxiety-related behavior. Since the single *mPer1* and *mPer2* deficient lines showed inconsistent or nonsignificant results in these measures, this suggests that each protein is likely able to compensate to some extent for the loss of the other.

Mutations in the Period genes do not alter the locomotor response to novelty

Mice with a mutation in the *Clock* gene are hyperactive in response to a novel environment (Roybal et al., 2007). Thus, we wanted to determine if mice with a mutation in either *mPer1*, *mPer2*, or *mPer1;mPer2* have any differences in novelty-induced activity. Mice were placed in an unfamiliar chamber for two hours and beam breaks were counted in 5 minute bins. We found that loss of *Period* gene function did not lead to a significant change in total locomotor activity across the 2 hour span (Figure A.2) in any of the lines tested. These results suggest that *mPer1* and *mPer2* are not involved in the regulation of the locomotor response to novelty.

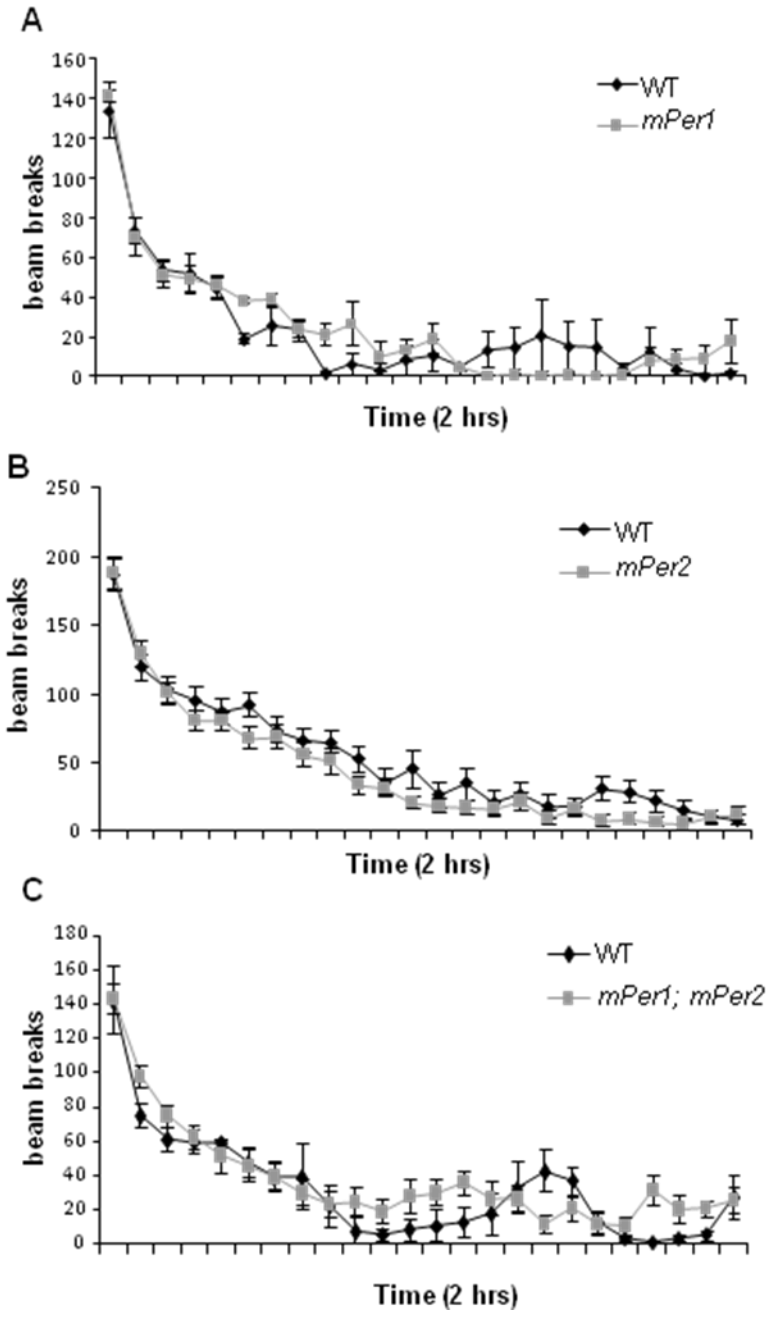

Figure A.2 Mice with mutations in the *Per* genes have a normal locomotor response to novelty. *mPer1* mutant mice (A) *mPer2* mutant mice (B) and *mPer1;mPer2* mutant mice (C) all were not significantly different than wild type mice in the locomotor response to a novel environment over 2hrs (n=13-24).

These results also confirm that the decreased exploratory behavior seen in the anxiety-related tests are not due to an overall decrease in locomotor activity.

Chronic social defeat and antidepressant treatment alter Period gene expression in the NAc

Since mice with mutations in both *Per* genes have significant behavioral phenotypes in anxiety-related measures, we wanted to know if chronic stress or anxioloytic/ antidepressant treatment would alter *Per* gene expression in the nucleus accumbens (NAc). We decided to employ a social defeat paradigm in which mice are subjected to attacks by an aggressive mouse once a day for 10 days. They are also housed in an environment where they can see and smell the aggressor at all times during the 10 day protocol. Previous studies have found that in the majority of mice, this paradigm leads to a profound increase in social avoidance behavior and anxiety-related behavior which is extremely long lasting, but can be reversed with chronic imipramine treatment (Berton et al., 2006; Tsankova et al., 2006). We chose to examine changes in expression in the nucleus accumbens (NAc) which is highly involved in both depression and anxiety-related behavior because previous studies have found that social defeat leads to specific gene expression changes in this region (Krishnan et al., 2007; Nestler and Carlezon, 2006). Furthermore, *Per* gene expression is strong in striatal regions, and can be altered in these regions by other behavioral paradigms such as those that employ chronic exposure to drugs of abuse (Iijima et al., 2002; Nikaido et al., 2001).

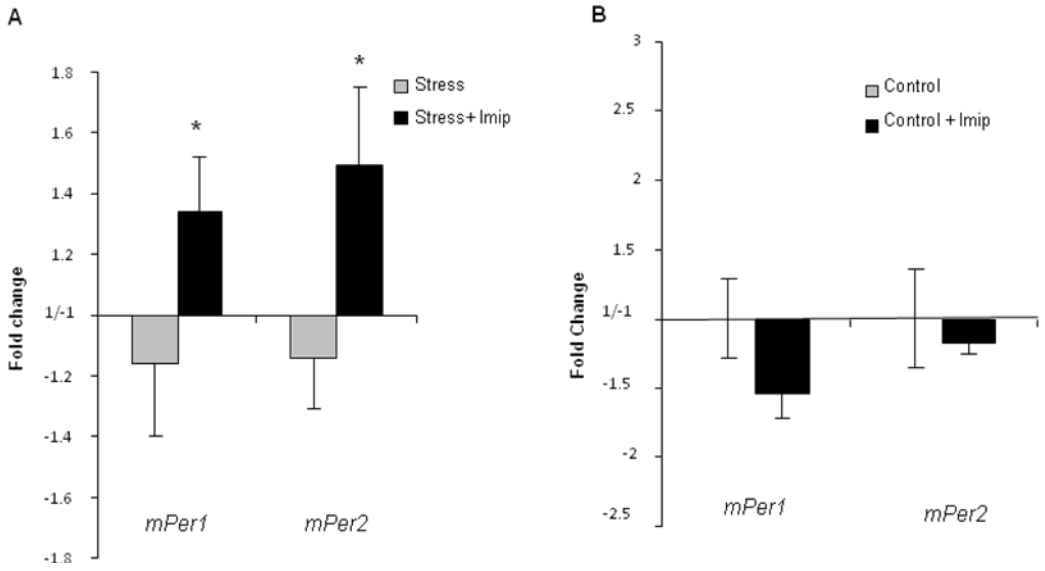

Figure A.3 Chronic stress followed by imipramine treatment leads to an increase in *mPer1* and *mPer2* expression in the NAc. WT mice were subjected to 10 days of social defeat stress followed by 28 days of vehicle or imipramine treatment. Control mice were only subjected to the 28 days of vehicle or imipramine but not the stress. *mPer1* and *mPer2* mRNA expression was significantly increased in the NAc following chronic stress and imipramine treatment (A) but not with imipramine treatment alone (B). (n=5 *p<0.05). The decrease in *mPer1* and *mPer2* expression in the vehicle treated group either with or without defeat stress was not significant.

After 10 days of defeat followed by 28 days of vehicle or imipramine treatment, we found that mice which had vehicle treatment had no significant changes in expression of either *mPer1* or *mPer2* (Figure A.3). However, mice that received imipramine treatment following defeat had a significant upregulation of both *mPer1* and *mPer2* (Figure A.3A). Interestingly, control mice which did not experience the social defeat but did have chronic imipramine treatment had no significant changes in *mPer1* or *mPer2* (Figure A.3B). This suggests that the induction of the *Per* genes with anxiolytic/ antidepressant treatment is specific to animals in an anxious/depressed state.

Disruption in PER1/PER2 function leads to profound gene expression changes in the NAc that suggest a role in stress responsiveness

The PER proteins are known to influence gene transcription via binding to other transcription factors (Ko and Takahashi, 2006). We wanted to determine the changes in gene expression in the NAc that might underlie the anxiogenic phenotypes of the *mPer1;mPer2* mutant mice, and identify any molecular pathways that seem to be controlled by these proteins. Using Affymetrix microarrays comparing NAc tissue from *mPer1;mPer2* mutant mice versus WT controls we found a number of genes to be differentially regulated in this region (Suppl. Table A.1). Many of the fold changes were extremely high (up to a fold change of 163) compared to the modest changes in expression (less than 2 fold) most often identified in microarray studies of the NAc (Berton et al., 2006; McClung and Nestler, 2003). Moreover, Gene Set Enrichment Analysis (GSEA)

(Mootha et al., 2003; Subramanian et al., 2005) found the highest concordance with genes involved in oxidative stress, macrophage function, response to DNA breaks and major depression (Suppl. Table A.2), as well as a number of genes found to be differentially regulated in various forms of cancer . These results demonstrate the importance of the *Period* genes in the regulation of gene expression in the NAc and point towards a crucial role for these genes in the cellular response to stress.

Knockdown of mPer1 and mPer2 in the nucleus accumbens contributes to anxiety-related behavior

To determine if a knock-down of *mPer1* and *mPer2* gene expression specifically in the NAc would be sufficient to increase anxiety, we employed an AAV virus with a short hairpin RNA which targeted a common sequence in both *mPer1* and *mPer2*. This leads to a decrease in the expression of both genes via RNA interference (RNAi) mechanisms. In mouse embryonic fibroblast cells, this AAV-shRNA led to large decreases in *mPer1* and *mPer2* expression (7.2 fold and 13.3 fold respectively, data not shown) when compared to expression of an AAV-scrambled control shRNA (AAV-scr) which does not match the sequence of any known gene. Infection with the AAV-*mPer1/mPer2* shRNA virus in the NAc of intact mice lead to a more modest 3 fold decrease in *mPer1* and a 2.4 fold decrease in *mPer2* compared with controls (Figure A.4A,B,C). To ensure that this decrease was specific to *mPer1* and *mPer2* we measured levels of *mPer3* and found no change in expression (data not shown). After waiting two weeks

for full viral expression in the NAc, we subjected AAV-*mPer1/mPer2* shRNA infected mice and mice expressing the AAV-Scr shRNA to the elevated plus maze and the open field. Even with this modest decrease in *mPer1* and *mPer2* expression in the NAc, we saw a significant decrease in the frequency of open arm entries in the EPM and time spent in the center of the open field (Figure A.4D,E). These data suggest that *mPer1* and *mPer2* expression in the NAc is important in the regulation of anxiety-related behavior.

Discussion

Our results show that the *Per* genes are involved in the regulation of anxiety-related behavior. Mice lacking both *mPer1* and *mPer2* have a robust increase in anxiety-related behavior while the single gene mutants display inconsistent results across tests with mice lacking a functional *mPer1* gene having greater anxiety in only a subset of measures. This might suggest a slightly greater role for *mPer1* over *mPer2* in anxiety, though the two genes appear to have some overlap in function. A recent study by Dong et al., found that *mPer1* mutant mice show enhanced alcohol consumption following social defeat stress relative to wild type mice (Dong et al., 2011). Moreover, a SNP in the *hPer1* promoter which leads to lowered cortisol-induced *Per1* transcription, was associated with psychosocial adversity and drinking in adolescents (Dong et al., 2011). In line with our results, these results suggest that lowered *Per* gene expression is associated with a heightened and anxiogenic response to stress.

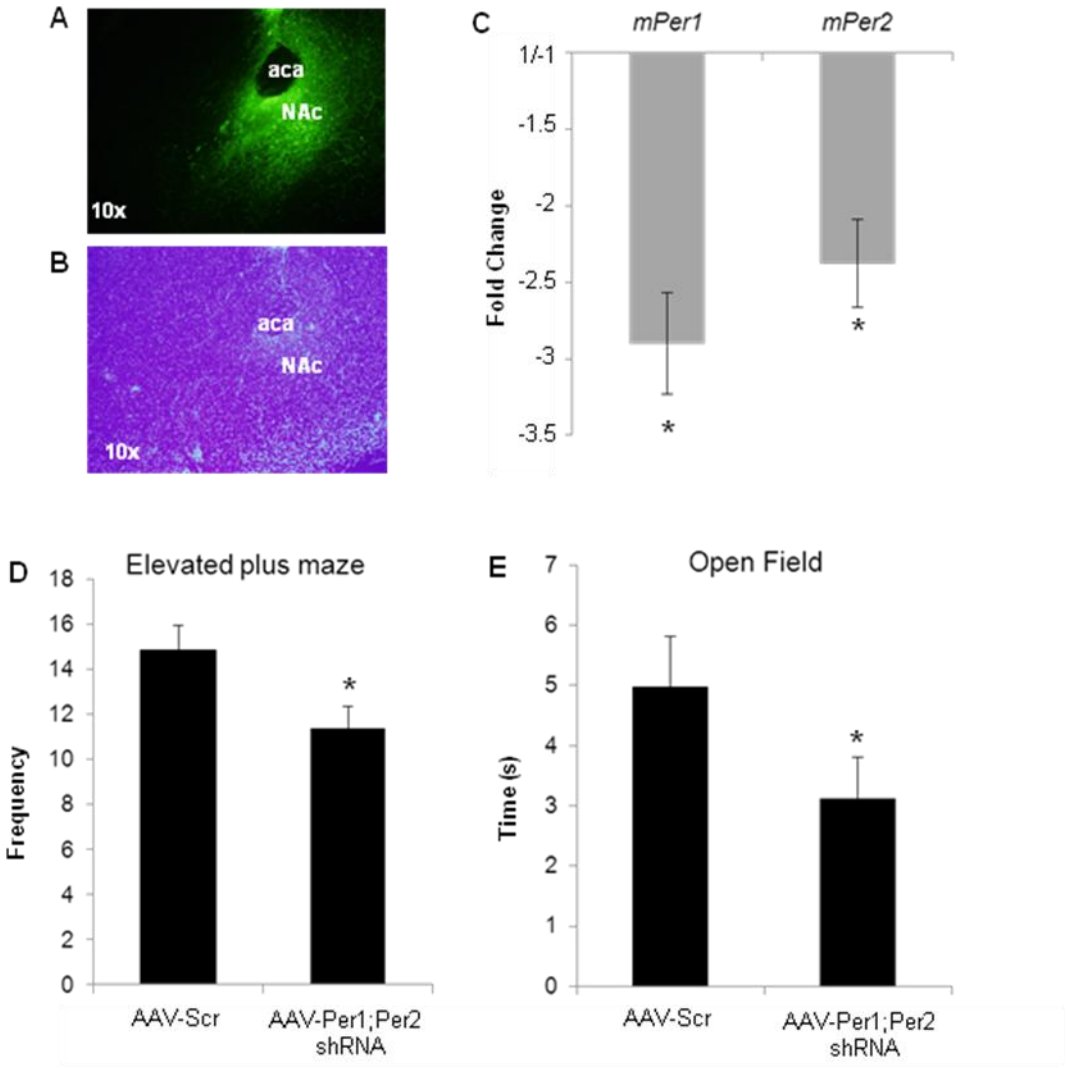

Figure A.4. Knock-down of *mPer1* and *mPer2* in the NAc increases anxiety. Mice were infused with the AAV-mPer1;mPer2 shRNA or AAV-Scr into the NAc and behavior was measured 2 weeks later. (A) Representative image showing viral expression in the NAc (B) DAPI staining of the same slice (C) AAV-mPer1;mPer2 infusion leads to a significant knock-down of mPer1 and mPer2 expression in the NAc. AAV-mPer1;mPer2shRNA NAc infected mice had a decreased frequency in the open arms of the EPM (D) and spent less time in the center of the open field (E) (n=10-11, *P<0.05).

It is interesting that the *mPer1;mPer2* mutant mice have an increase in anxiety-related behavior while mice with a mutation in the *Clock* gene have a decrease in anxiety-related behavior (Roybal et al., 2007). The *Clock* mutant mice are also hyperactive in response to novelty while the *Per* gene mutants are normal in these measures. These results show that disruption in any of the core circadian genes does not result in a common behavioral profile. CLOCK and PER proteins have opposing activity in the circadian loop with PER proteins acting as inhibitors of CLOCK. Therefore perhaps an opposing behavioral phenotype in these measures is not surprising if it is dependent upon CLOCK activity. Interestingly, SCN lesions lead to an antidepressant effect in measures of behavioral despair, but no change in anxiety related behavior (Engelmann et al., 1998; Tataroglu et al., 2004; Tuma et al., 2005). This suggests that the anxiogenic effects seen in the m*Per1;mPer2* mutant mice are due to a lack of *Per* gene function in other brain regions outside of the SCN.

The nucleus accumbens is a known center of emotional regulation and many studies have implicated this region in the response to stress and development of anxiety (Barrot et al., 2002; Krishnan and Nestler, 2008; Nestler and Carlezon, 2006). *Per* gene expression appears to be particularly important in the NAc since we find that local knock-down in this region is sufficient to increase anxiety-related behavior. Following chronic social defeat stress there was a trend towards decreased *Per* gene expression in the NAc, though these results did not reach significance. This tissue was taken 28 days after the end of the 10 day defeat protocol. It is possible that there was a difference that reached

significance at an earlier time point. However, we did find a significant increase in *Per1* and *Per2* following chronic imipramine only in animals that had experienced social defeat. These results demonstrate that there are molecular adaptations that occur in response to chronic stress which are anxiogenic and the *Per* genes may be involved in the mechanism by which imipramine is able to reverse these changes and restore normal behavior. Interestingly, a recent study by Koresh and colleagues found that expression of *mPer1* and *mPer2* were elevated in the hippocampus, frontal cortex and SCN eight days after exposure to predator scent stress in animals with an "extreme" (i.e. PTSD-like) behavioral response (Koresh et al., 2011). Immediate treatment with agomelatine reversed these changes. This suggests that there are wide spread disruptions in *Per* gene expression throughout the brain following stress, and whether the changes are up or down might be specific to the region of the brain, amount of time following stress, and the time of day in which changes are measured. Future studies will determine if alterations in *Per* gene expression in these other regions results in altered anxiety-like behavior.

Microarray analysis of the NAc of *mPer1;mPer2* mutant mice versus wild type controls identified large changes in gene expression overall, and specifically changes in groups of genes that are responsive to DNA breaks, oxidative stress and other metabolic processes. These results show the importance of the *Per* genes in normal cellular function and the response to cellular stress. Interestingly, acute psychosocial stress in humans is sufficient to elicit changes in

cellular stress responses in the immune system, cell cycle regulation, and cell death pathways (Nater et al., 2009).

A number of genes involved in tumor growth and differentiation were also identified which was not unexpected given the numerous reports of altered *Per* gene expression in various tumors, and the important role of the PER proteins as tumor suppressors (Fu and Lee, 2003; Yu and Weaver, 2011). Notably, ten of the genes altered in the *mPer1;mPer2* mutant mice were also found to be downregulated in the temporal cortex of human postmortem tissue from subjects with major depression (Aston et al., 2005). Anxiety is often highly co-morbid with depression, and future studies will determine the role of the *Per* genes in the NAc in depression-related behavior.

In summary, *mPer1* and *mPer2* in the NAc appear to be centrally involved in the response to stress and development of anxiety. Our results also suggest that an increase in these proteins in the NAc could be beneficial in the reversal of anxiety-related behavior following chronic stress. Thus the Per genes represent new potential therapeutic targets for the treatment of anxiety disorders.

Supplementary Table A.1 All significant gene expression changes in the Nac of mPer1;mPer2 mutant mice versus controls

Gene Symbol	Fold Change	Gene Name
UPREGULATED		
Pdxdc1	163.07986	pyridoxal-dependent decarboxylase domain containing 1
Skiv2l2	22.703724	superkiller viralicidic activity 2-like 2 (S. cerevisiae)
Tmem87a	22.247902	transmembrane protein 87A
Ints10	10.814575	integrator complex subunit 10
Pldn	10.153809	pallidin
Zfp64	5.197372	zinc finger protein 64
Kcnj9	4.921765	potassium inwardly-rectifying channel, subfamily J, member 9
Pla2g4e	4.753624	phospholipase A2, group IVE
Mamdc2	4.653901	MAM domain containing 2
H2-Bl	4.0462403	histocompatibility 2, blastocyst
Esco1	3.9394853	establishment of cohesion 1 homolog 1 (S. cerevisiae)
Casc4	3.892533	cancer susceptibility candidate 4
Usp14	3.790768	ubiquitin specific peptidase 14
Med1	3.658584	mediator complex subunit 1
Tsc22d1	3.4655244	TSC22-related inducible leucine zipper 1b (Tilz1b)
Ocel1	3.4643505	occludin/ELL domain containing 1
Zbtb16	3.3775492	zinc finger and BTB domain containing 16
Zfp398	3.2163012	zinc finger protein 398
Armc1	3.105523	armadillo repeat containing 1
Supt16h	3.1010184	suppressor of Ty 16 homolog (S. cerevisiae)
Ramp1	3.053394	receptor (calcitonin) activity modifying protein 1
Atp1a2	3.044215	ATPase, Na+/K+ transporting, alpha 2 polypeptide
Tle4	3.00081	transducin-like enhancer of split 4, homolog of Drosophila E(spl)
Wdfy1	2.9433482	WD repeat and FYVE domain containing 1
D5Ertd798e	2.9188883	DNA segment, Chr 5, ERATO Doi 798, expressed
Abhd1	2.8570073	abhydrolase domain containing 1
Lcorl /// LOC100046011	2.8155901	ligand dependent nuclear receptor corepressor-like /// hypothetical protein LOC100046011
Wdfy1	2.8040495	WD repeat and FYVE domain containing 1
Tsen2	2.7217097	tRNA splicing endonuclease 2 homolog (S. cerevisiae)
Med1	2.6870406	mediator complex subunit 1
Gm129	2.6608152	gene model 129, (NCBI)
Mpp7	2.6464226	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)
Plvap	2.5619984	plasmalemma vesicle associated protein
Folh1	2.5427	folate hydrolase
Adcy7	2.4037695	adenylate cyclase 7
Cnnm1	2.380429	cyclin M1
Gzmk	2.3771994	granzyme K
Slc15a2	2.3724248	solute carrier family 15 (H+/peptide transporter), member 2
Mpp7	2.332721	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)
Rbm45	2.2818651	RNA binding motif protein 45
Supt7l	2.27437	suppressor of Ty 7 (S. cerevisiae)-like
Kcnb1	2.2742388	potassium voltage gated channel, Shab-related subfamily, member 1
Psip1	2.2400606	PC4 and SFRS1 interacting protein 1 (Psip1), mRNA
Alad /// LOC100046072	2.2390966	aminolevulinate, delta-, dehydratase /// similar to aminolevulinate, delta-, dehydratase
Rpgrip1	2.2139251	retinitis pigmentosa GTPase regulator interacting protein 1

Armc9	2.194753	armadillo repeat containing 9
Spry2	2.1933522	sprouty homolog 2 (Drosophila)
Iqcf1	2.1930766	IQ motif containing F1
Mysm1	2.178694	myb-like, SWIRM and MPN domains 1
Ugcgl2	2.1776702	UDP-glucose ceramide glucosyltransferase-like 2
Rragd	2.168193	Ras-related GTP binding D
Lsm12	2.1629736	LSM12 homolog (S. cerevisiae)
Pigz	2.1594698	phosphatidylinositol glycan anchor biosynthesis, class Z
EG623818 /// Hmbs	2.1117568	predicted gene, EG623818 /// hydroxymethylbilane synthase
Apod /// LOC100047583	2.102918	apolipoprotein D /// similar to apolipoprotein D
Ppwd1	2.0985777	peptidylprolyl isomerase domain and WD repeat containing 1
Fmo1	2.0854836	flavin containing monooxygenase 1
Pyroxd2	2.0730407	pyridine nucleotide-disulphide oxidoreductase domain 2
Rbm39	2.0698643	RNA binding motif protein 39
Gprc5b	2.0694625	G protein-coupled receptor, family C, group 5, member B
Cntn3	2.0639896	contactin 3
Nrd1	2.0595195	nardilysin, N-arginine dibasic convertase, NRD convertase 1
Marveld2	2.0464137	MARVEL (membrane-associating) domain containing 2
Fst	2.031238	follistatin
Zfp69	2.0211785	zinc finger protein 69
Pitpnc1	2.0189035	phosphatidylinositol transfer protein, cytoplasmic 1
Edil3	2.0163221	Del1 minor splice variant (Del1)
Hist3h2ba	1.9881265	histone cluster 3, H2ba
Crkrs	1.9570255	CDC2-related kinase, arginine/serine-rich
Fam20b	1.9541714	Family with sequence similarity 20, member B, mRNA (cDNA clone MGC:36624 IMAGE:5352660)
Rbm39	1.9500977	RNA binding motif protein 39
Lsm12	1.9348234	LSM12 homolog (S. cerevisiae)
Nr1d2	1.880898	nuclear receptor subfamily 1, group D, member 2
Smg1	1.8427855	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)
Epb4.1l1	1.8404436	erythrocyte protein band 4.1-like 1
Ifit3	1.8122317	interferon-induced protein with tetratricopeptide repeats 3
Kcnj10	1.7845392	potassium inwardly-rectifying channel, subfamily J, member 10
Adcy7	1.7844566	adenylate cyclase 7
Cdh7	1.76733	cadherin 7, type 2
Nnt	1.739978	nicotinamide nucleotide transhydrogenase
Lyplal1	1.7392213	lysophospholipase-like 1
Wdfy1	1.7313237	WD repeat and FYVE domain containing 1
Rfesd	1.7194365	Rieske (Fe-S) domain containing
Mthfd1l	1.7133402	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
Gpr137c	1.69243	G protein-coupled receptor 137C
Sfrp1	1.6748738	secreted frizzled-related protein 1
Mpp3	1.6490617	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)
Slc14a1	1.6451137	solute carrier family 14 (urea transporter), member 1
Hey2	1.6422431	hairy/enhancer-of-split related with YRPW motif 2
Sypl	1.6406695	synaptophysin-like protein
Dynlt1d /// Tmem181	1.6370541	dynein light chain Tctex-type 1D /// transmembrane protein 181
Nqo1	1.6196786	NAD(P)H dehydrogenase, quinone 1
Pi4k2b	1.6164364	phosphatidylinositol 4-kinase type 2 beta
Ptgfrn	1.6036649	prostaglandin F2 receptor negative regulator
F2r	1.6014299	coagulation factor II (thrombin) receptor
Chd2	1.5989904	chromodomain helicase DNA binding protein 2
Sfi1	1.5978068	Sfi1 homolog, spindle assembly associated (yeast)
Tmem181	1.5966526	transmembrane protein 181

Orc3l	1.5856255	origin recognition complex, subunit 3-like (S. cerevisiae)
Rpl17	1.5852182	ribosomal protein L17
Bxdc2	1.5758436	brix domain containing 2
Rgs16	1.5758215	regulator of G-protein signaling 16
Fam149a	1.5745534	family with sequence similarity 149, member A
LOC640502 /// Uap1	1.5733553	similar to UDP-N-acetylhexosamine pyrophosphorylase /// UDP-N-acetylglucosamine pyrophosphorylase 1
Wdr16	1.571068	WD repeat domain 16
LOC433064 /// Ppih	1.5686675	similar to peptidyl prolyl isomerase H /// peptidyl prolyl isomerase H
Ppm2c	1.5682639	protein phosphatase 2C, magnesium dependent, catalytic subunit
Zwint	1.5582784	ZW10 interactor
Rrad	1.5579627	Ras-related associated with diabetes
Sfxn4	1.5543066	sideroflexin 4
Sympk	1.5542725	PREDICTED: Mus musculus symplekin (Sympk), mRNA
Exd1	1.5517102	exonuclease 3'-5' domain containing 1
Kcnq1ot1	1.5399141	KCNQ1 overlapping transcript 1
Lpl	1.5310428	lipoprotein lipase
Inpp4b	1.5197984	inositol polyphosphate-4-phosphatase, type II
Rdh13	1.5181713	retinol dehydrogenase 13 (all-trans and 9-cis)
Pm20d1	1.5174752	peptidase M20 domain containing 1
Pclo	1.5100782	piccolo (presynaptic cytomatrix protein)
Mut	1.4988123	methylmalonyl-Coenzyme A mutase
Sp1	1.4979638	trans-acting transcription factor 1
Glo1	1.4886348	glyoxalase 1
Dnaic1	1.4851202	dynein, axonemal, intermediate chain 1
LOC100048247	1.4849001	similar to polycomb group ring finger 5
Ptchd1	1.4807725	patched domain containing 1
Itm2b	1.4799745	integral membrane protein 2B
Mut	1.4730355	methylmalonyl-Coenzyme A mutase
Limch1	1.4726206	LIM and calponin homology domains 1 (Limch1), mRNA
Itch	1.4720265	itchy, E3 ubiquitin protein ligase
Nupl1	1.4676307	nucleoporin like 1
Ccr6	1.4660307	chemokine (C-C motif) receptor 6
Svil	1.4626781	supervillin
Enox1	1.4575627	ecto-NOX disulfide-thiol exchanger 1
Zbtb10	1.449157	zinc finger and BTB domain containing 10
Tctex1d2	1.4478508	Tctex1 domain containing 2
Ston1	1.4419018	Stonin 1 (Ston1), mRNA
Wtap	1.4408981	Wilms' tumour 1-associating protein
Zc3h12c	1.4389921	zinc finger CCCH type containing 12C
Tprkb	1.4375784	Tp53rk binding protein
C030033M12Rik /// Rsf1	1.4370954	RIKEN cDNA C030033M12 gene /// remodeling and spacing factor 1
Stk35	1.4360473	serine/threonine kinase 35
Mllt3	1.4359876	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3
Lrrc4c	1.4345628	Leucine rich repeat containing 4C, mRNA (cDNA clone MGC:106508 IMAGE:30617370)
Gja1	1.4341168	gap junction protein, alpha 1
Ercc2	1.4303242	Excision repair cross-complementing rodent repair deficiency, complementation group 2, mRNA (cDNA clone MGC:27532 IMAGE:4458839)
Slc37a4	1.4295944	solute carrier family 37 (glucose-6-phosphate transporter), member 4
Me2	1.4267718	malic enzyme 2, NAD(+)-dependent, mitochondrial
Gspt2	1.4244307	G1 to S phase transition 2
Fbxo3	1.4211305	F-box protein 3
Slc14a1	1.4178236	solute carrier family 14 (urea transporter), member 1

Slc7a11	1.4165592	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11
Arhgap4	1.415351	Rho GTPase activating protein 4
Ifi30	1.4006002	interferon gamma inducible protein 30
Rbm8a	1.3990426	RNA binding motif protein 8a
Pgap1	1.3952152	post-GPI attachment to proteins 1
Peg3	1.3952061	paternally expressed 3
Usf1	1.39364	upstream transcription factor 1
Stxbp4	1.3926165	syntaxin binding protein 4
Sf4	1.3920588	splicing factor 4
Ldhd	1.391458	lactate dehydrogenase D
Zfp295	1.3886931	zinc finger protein 295
Gmip	1.3882145	Gem-interacting protein
Rab3gap1	1.3871458	RAB3 GTPase activating protein subunit 1
Dbt	1.3871008	dihydrolipoamide branched chain transacylase E2
Slc25a36	1.3864928	solute carrier family 25, member 36
Fam62b	1.3800949	family with sequence similarity 62, member B
Apol8	1.3796602	apolipoprotein L 8
Mocs1	1.378669	molybdenum cofactor synthesis 1
Pnma5	1.3767962	paraneoplastic antigen family 5
Ppm1k	1.3766019	protein phosphatase 1K (PP2C domain containing)
Clptm1	1.3761804	cleft lip and palate associated transmembrane protein 1
Cercam	1.3751626	cerebral endothelial cell adhesion molecule
Zik1	1.3743591	zinc finger protein interacting with K protein 1
LOC100045567 /// Pnp1	1.3718723	similar to purine nucleoside phosphorylase /// purine-nucleoside phosphorylase 1
Eif2s2	1.3717732	eukaryotic translation initiation factor 2, subunit 2 (beta)
Pdia3	1.3708751	protein disulfide isomerase associated 3
Epha4	1.3708341	Eph receptor A4
Amigo2	1.3694645	adhesion molecule with Ig like domain 2
Hnrnpc /// OTTMUSG00000008006	1.3682587	heterogeneous nuclear ribonucleoprotein C /// predicted gene, OTTMUSG00000008006
Rnls	1.3676354	renalase, FAD-dependent amine oxidase
Socs4	1.3670021	suppressor of cytokine signaling 4
Prkg2	1.3640962	protein kinase, cGMP-dependent, type II
Rc3h2	1.3616189	ring finger and CCCH-type zinc finger domains 2
Wdr62	1.360827	WD repeat domain 62
DOWNREGULATED		
Rps9	37.406506	ribosomal protein S9
Ccl21a /// Ccl21b /// Ccl21c	22.101341	chemokine (C-C motif) ligand 21A /// chemokine (C-C motif) ligand 21B /// chemokine (C-C motif) ligand 21C (leucine)
Tmem40	21.573027	transmembrane protein 40
Scn2b	19.646719	sodium channel, voltage-gated, type II, beta
Cd99l2	16.75769	CD99 antigen-like 2
Pdxdc1	15.909773	pyridoxal-dependent decarboxylase domain containing 1
Cript	13.1127615	cysteine-rich PDZ-binding protein
Cd99l2	12.884508	CD99 antigen-like 2
Mtmr7	11.385848	myotubularin related protein 7
Rmst	10.657746	rhabdomyosarcoma 2 associated transcript (non-coding RNA)
Pnma2	10.026945	paraneoplastic antigen MA2
Trp53bp1	9.9271755	transformation related protein 53 binding protein 1
Slc15a2	9.005051	solute carrier family 15 (H+/peptide transporter), member 2
Hps1	8.533	Hermansky-Pudlak syndrome 1 homolog (human)
Trp53bp1	8.276409	transformation related protein 53 binding protein 1
Ang	7.470926	angiogenin, ribonuclease, RNase A family, 5
Ppcdc	6.9503965	phosphopantothenoylcysteine decarboxylase

BC031748	6.8134575	CDNA sequence BC031748 (BC031748), mRNA
Tia1	6.726292	cytotoxic granule-associated RNA binding protein 1
Pdxdc1	6.501792	Pyridoxal-dependent decarboxylase domain containing 1 (Pdxdc1), transcript variant 1, mRNA
Adi1	6.208035	acireductone dioxygenase 1
Ang	6.0214067	angiogenin, ribonuclease, RNase A family, 5
Fmn2	5.740571	formin 2
Myo7a	5.461564	myosin VIIA
Tmem40	5.2910075	transmembrane protein 40
Spata5l1	5.0570335	spermatogenesis associated 5-like 1
Adamts4	4.8463306	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4
Prdx2	4.7878447	peroxiredoxin 2
Atp2c1	4.646063	ATPase, Ca++-sequestering
Mga	4.492594	MAX gene associated
Scmh1	4.3125167	Sex comb on midleg homolog 1 (Scmh1), mRNA
Cdh7	3.7953184	cadherin 7, type 2
Prkaa2	3.7547832	protein kinase, AMP-activated, alpha 2 catalytic subunit
Rpgrip1	3.5593228	retinitis pigmentosa GTPase regulator interacting protein 1
Atm	3.4730444	ataxia telangiectasia mutated homolog (human)
Pi4k2a	3.4565237	phosphatidylinositol 4-kinase type 2 alpha
Kctd14	3.4437954	potassium channel tetramerisation domain containing 14
Lman1	3.418434	lectin, mannose-binding, 1
Dact3	3.3224185	dapper homolog 3, antagonist of beta-catenin (xenopus)
Caskin1	3.1503828	CASK interacting protein 1
Prkaa2	3.1296315	protein kinase, AMP-activated, alpha 2 catalytic subunit
Pdxdc1	3.1057012	pyridoxal-dependent decarboxylase domain containing 1
Zfp593	2.977452	zinc finger protein 593
Gp1ba	2.868199	glycoprotein 1b, alpha polypeptide
Ppp1r3e	2.8280532	protein phosphatase 1, regulatory (inhibitor) subunit 3E
Dusp12	2.8086545	dual specificity phosphatase 12
Elavl1	2.7999382	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)
Acaca /// EG382567	2.7313104	acetyl-Coenzyme A carboxylase alpha /// predicted gene, EG382567
Nav1	2.7038946	neuron navigator 1
Cryab	2.6720061	crystallin, alpha B
Cttnbp2	2.5762818	cortactin binding protein 2
Chic1	2.5659368	Cysteine-rich hydrophobic domain 1, mRNA (cDNA clone MGC:170357 IMAGE:8861752)
Hisppd2a	2.5564866	histidine acid phosphatase domain containing 2A
Mkks	2.516239	McKusick-Kaufman syndrome protein
Mpeg1	2.4869516	macrophage expressed gene 1
Tmem40	2.4140115	transmembrane protein 40
Tm2d2	2.3754737	TM2 domain containing 2
Cand2	2.345365	cullin-associated and neddylation-dissociated 2 (putative)
Capn3	2.295221	calpain 3
Paqr7	2.277379	Progestin and adipoQ receptor family member VII (Paqr7), mRNA
Tulp4	2.2697606	tubby like protein 4
Map3k7	2.2029471	mitogen-activated protein kinase kinase kinase 7
Car8	2.1094012	carbonic anhydrase 8
Zcchc3	2.100542	zinc finger, CCHC domain containing 3
Rps6	2.0650408	ribosomal protein S6
Spag9	2.0382607	sperm associated antigen 9
Itch	2.033723	itchy, E3 ubiquitin protein ligase
Cep192	2.0272698	Premature mRNA for mKIAA1569 protein
Cxcl13	2.0024102	chemokine (C-X-C motif) ligand 13
Samd4	1.9869547	sterile alpha motif domain containing 4

Prdm16	1.9658161	PR domain containing 16
Ggcx	1.9560659	gamma-glutamyl carboxylase
Ccl27a	1.9344465	chemokine (C-C motif) ligand 27A
Cryab	1.934077	crystallin, alpha B
Trim34	1.9124783	tripartite motif-containing 34
Map2k7	1.9054939	mitogen-activated protein kinase kinase 7
Gjb1	1.897576	gap junction protein, beta 1
Ccl6	1.8779497	chemokine (C-C motif) ligand 6
Naip5	1.8731921	NLR family, apoptosis inhibitory protein 5
Ccl27a	1.869179	chemokine (C-C motif) ligand 27A
Prr18	1.8563688	proline rich region 18
Nav1	1.8495973	neuron navigator 1
Fbxo44	1.8391204	F-box protein 44
Dcp1b	1.8389121	DCP1 decapping enzyme homolog b (S. cerevisiae)
Ncapd2	1.8312722	non-SMC condensin I complex, subunit D2
Fem1a	1.8288018	feminization 1 homolog a (C. elegans)
Trf	1.8201282	transferrin
Slc24a2	1.8180546	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
Esd	1.8077459	Esterase D/formylglutathione hydrolase (Esd), mRNA
LOC100048604 /// Ninj2	1.8072416	similar to ninjurin2 /// ninjurin 2
Pllp	1.7969198	plasma membrane proteolipid
Fndc3a	1.787043	fibronectin type III domain containing 3A
Gadd45gip1	1.7820506	growth arrest and DNA-damage-inducible, gamma interacting protein 1
Aldh7a1	1.7735661	aldehyde dehydrogenase family 7, member A1
Lgi3	1.7666689	leucine-rich repeat LGI family, member 3
Stxbp2	1.7515991	syntaxin binding protein 2
Klf12	1.7499818	Kruppel-like factor 12
Pgpep1	1.7370445	pyroglutamyl-peptidase I
H2-T10 /// H2-T17 /// H2-T22 /// H2-T9	1.7369473	histocompatibility 2, T region locus 10 /// histocompatibility 2, T region locus 17 /// histocompatibility 2, T region locus 22 /// histocompatibility 2, T region locus 9
Lgals7 /// Samd4	1.7175795	lectin, galactose binding, soluble 7 /// sterile alpha motif domain containing 4
Arsk	1.7149282	arylsulfatase K
Rad23a	1.7131382	RAD23a homolog (S. cerevisiae)
Frag1	1.7081468	FGF receptor activating protein 1
Stk25	1.7035123	serine/threonine kinase 25 (yeast)
Mark4	1.7030361	MAP/microtubule affinity-regulating kinase 4
Rpp25	1.6774338	ribonuclease P 25 subunit (human)
Kctd14	1.673872	potassium channel tetramerisation domain containing 14
Dbi	1.6634966	diazepam binding inhibitor
Polr1a	1.6590286	polymerase (RNA) I polypeptide A
Lmo1	1.6571361	LIM domain only 1
Coro2b	1.6556733	coronin, actin binding protein, 2B
Dhdds	1.6428918	dehydrodolichyl diphosphate synthase
Cntfr	1.642671	ciliary neurotrophic factor receptor
Nos1ap	1.6373652	nitric oxide synthase 1 (neuronal) adaptor protein
Nnmt	1.619879	nicotinamide N-methyltransferase
Galm	1.607401	galactose mutarotase
Pop4	1.5917711	processing of precursor 4, ribonuclease P/MRP family, (S. cerevisiae)
Klhdc9	1.5811667	kelch domain containing 9
Rab8a	1.5726717	RAB8A, member RAS oncogene family (Rab8a), mRNA
Vangl2	1.5709174	vang-like 2 (van gogh, Drosophila)
Fads6	1.5654644	fatty acid desaturase domain family, member 6
Slc25a19	1.563225	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19

Nlrx1	1.551315	NLR family member X1
Jmjd7	1.5492492	jumonji domain containing 7
Polr1a	1.5278773	polymerase (RNA) I polypeptide A
Mthfd2	1.5251637	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase
Tmem19	1.5234618	transmembrane protein 19
Cacnb1	1.5218232	calcium channel, voltage-dependent, beta 1 subunit
Odz4	1.5189627	odd Oz/ten-m homolog 4 (Drosophila)
Ltbp4	1.5178305	latent transforming growth factor beta binding protein 4
Myg1	1.5112839	melanocyte proliferating gene 1
Abcf1	1.5109109	ATP-binding cassette, sub-family F (GCN20), member 1
D6Wsu116e	1.5108125	DNA segment, Chr 6, Wayne State University 116, expressed
Oaf	1.5075518	OAF homolog (Drosophila)
Pon2	1.5072562	paraoxonase 2
Mrpl47	1.5071172	mitochondrial ribosomal protein L47
Zh2c2	1.5055702	zinc finger, H2C2 domain containing
Dbi	1.5017204	diazepam binding inhibitor
Ttpal	1.4988986	tocopherol (alpha) transfer protein-like
LOC627626	1.4961693	Similar to CG11212-PA, mRNA (cDNA clone MGC:184066 IMAGE:9088055)
Tradd	1.4951609	TNFRSF1A-associated via death domain
Dnajb5	1.4943137	DnaJ (Hsp40) homolog, subfamily B, member 5
Gstm7	1.4895003	glutathione S-transferase, mu 7
Dctd	1.4882056	dCMP deaminase
Frag1	1.4849548	FGF receptor activating protein 1
Yipf3	1.4784355	Yip1 domain family, member 3
Rad23a	1.4776158	RAD23a homolog (S. cerevisiae)
Rnf157	1.475672	ring finger protein 157
Snx5	1.4705888	sorting nexin 5
Ivns1abp	1.4669834	influenza virus NS1A binding protein
Zcchc11	1.4661901	zinc finger, CCHC domain containing 11
Rnf112	1.4660063	ring finger protein 112
Gatm	1.465518	glycine amidinotransferase (L-arginine:glycine amidinotransferase)
Chl1	1.461355	cell adhesion molecule with homology to L1CAM
Usp36	1.4610846	ubiquitin specific peptidase 36
Prl7c1	1.4584008	prolactin family 7, subfamily c, member 1
Amdhd2	1.451711	amidohydrolase domain containing 2
Bpnt1	1.4477185	bisphosphate 3'-nucleotidase 1
Dbi	1.4469645	diazepam binding inhibitor
Il15	1.4358268	interleukin 15
B4galt2	1.4349903	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 2
Oscar	1.4346075	osteoclast associated receptor
Cdc42ep1	1.4336593	CDC42 effector protein (Rho GTPase binding) 1
Mcart1	1.4310224	mitochondrial carrier triple repeat 1
Pkig	1.4298918	protein kinase inhibitor, gamma
Vars	1.4286813	valyl-tRNA synthetase
Sco2	1.4243373	SCO cytochrome oxidase deficient homolog 2 (yeast)
Hexdc	1.4233568	hexosaminidase (glycosyl hydrolase family 20, catalytic domain) containing
Myo6	1.4212536	myosin VI
Pon2	1.4211719	paraoxonase 2
Nit1	1.4199938	nitrilase 1
Prrt3	1.4157628	proline-rich transmembrane protein 3
Coro7	1.4122847	coronin 7
Sirt2	1.4120563	sirtuin 2 (silent mating type information regulation 2, homolog) 2 (S. cerevisiae)
Tnfrsf21	1.4036827	tumor necrosis factor receptor superfamily, member 21
Fbxl20	1.4023672	F-box and leucine-rich repeat protein 20
Fads3	1.3984222	fatty acid desaturase 3

Tec	1.3979338	tec protein tyrosine kinase
Fez2	1.3894987	fasciculation and elongation protein zeta 2 (zygin II)
Ccl28	1.3889949	chemokine (C-C motif) ligand 28
Slc20a1	1.3881154	solute carrier family 20, member 1
S100a16	1.385382	S100 calcium binding protein A16
S100a16	1.383751	S100 calcium binding protein A16
Dach2	1.3826895	dachshund 2 (Drosophila)
Slc9a3r1	1.382136	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1
Timp2	1.3813541	tissue inhibitor of metalloproteinase 2
Fgfrl1 /// LOC100046239	1.3806201	fibroblast growth factor receptor-like 1 /// similar to fibroblast growth factor receptor 5 beta
Zfp758	1.3784078	zinc finger protein 758
Trappc5	1.3782985	trafficking protein particle complex 5
Klf3 /// LOC100046855	1.376655	Kruppel-like factor 3 (basic) /// similar to BKLF
Gpr180	1.3754125	G protein-coupled receptor 180
Slc35a5	1.3729981	solute carrier family 35, member A5
Trim62	1.3727711	tripartite motif-containing 62
Slc25a22	1.3727641	solute carrier family 25 (mitochondrial carrier, glutamate), member 22
Pdk3	1.3726146	pyruvate dehydrogenase kinase, isoenzyme 3
Slc5a5	1.3724186	solute carrier family 5 (sodium iodide symporter), member 5
Cd276	1.3719692	CD276 antigen
Esd	1.3716016	esterase D/formylglutathione hydrolase
Uck2	1.3676988	uridine-cytidine kinase 2
Dbi	1.367662	diazepam binding inhibitor
Gsto1	1.3663433	glutathione S-transferase omega 1
Cyb5d2	1.3630785	cytochrome b5 domain containing 2

Supplementary Table A.2 Significant pathways (P<0.01) as identified by Gene Set Enrichment Analysis

Pathway	P_value	q_value	intersectional_gene_list
CHESLER_BRAIN_QTL_CIS	1.20E-08	5.01E-05	Aldh7a1, Slc15a2, Prdx2, Pdxdc1, Myo7a, Glo1, Ocel1, Abhd14a, Rgs16, Kcnj9, Sorl1, Folh1
COATES_MACROPHAGE_M1_VS_M2_UP	2.37E-06	0.004946	Gja1, Pldn, Abhd1, Kcnj10, Gatm, Glo1, Ocel1, Sfi1, Haus2, Lyplal1
CADWELL_ATG16L1_TARGETS_DN	2.79E-05	0.030876	Sfrp1, Slc15a2, Hist2h2bb, Skiv2l2, Haus2, Adi1, Arsk, Spata5l1
CHESLER_BRAIN_HIGHEST_GENETIC_VARIANCE	2.96E-05	0.030876	Aldh7a1, Prdx2, Pdxdc1, Ocel1, Kcnj9, Folh1
CADWELL_ATG16L1_TARGETS_UP	7.23E-05	0.051063	Lpl, Pldn, Tmem87a, Wdfy1, Kctd14, Fmn2, Rbm45, Exd1, Arsk
COATES_MACROPHAGE_M1_VS_M2_DN	7.34E-05	0.051063	Prdx2, Pdxdc1, Rpgrip1, Cript, Gprc5b, Ang, Tulp4, Arsk
BREDEMEYER_RAG_SIGNALING_NOT_VIA_ATM_UP	0.0001674	0.0974613	Lsm12, Smg1, Iqcf1, Mpp7, Rnf157, Slc20a1, Ccdc25
RAS_GTPASE_ACTIVATOR_ACTIVITY	0.0002022	0.0974613	Arhgap4, Cdc42ep2, Gmip, Dock4, Rab3gap1
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_AND_BRAIN_QTL_CIS	0.0002102	0.0974613	Prdx2, Pdxdc1, Myo7a, Med1, Glo1, Ocel1, Kcnj9
RHO_GTPASE_ACTIVATOR_ACTIVITY	0.0003108	0.1296617	Arhgap4, Cdc42ep2, Gmip, Dock4
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL_CIS	0.0008465	0.2990131	Prdx2, Pdxdc1, Med1, Dbi, Gatm, Glo1, Ocel1, Pon2, F2r
TURASHVILI_BREAST_DUCTAL_CARCINOMA_VS_DUCTAL_NORMAL_DN	0.0008601	0.2990131	Cryab, Sfrp1, Chl1, Rrad, Gatm, Trf, Atp1a2, Atp10d, Ints10, Ccl28, Mamdc2
AMUNDSON_RESPONSE_TO_ARSENITE	0.0010995	0.3058679	Eif2s2, Gucy1a3,

			Dnajb5, Klf12, Tle4, Chd2, Atp2c1, Cherp, Atp10d, Tulp4, Serpinh1
XU_CREBBP_TARGETS_DN	0.0011036	0.3058679	Per2, Epha4, Ocel1, Ercc2, Ccr6
TURASHVILI_BREAST_DUCTAL_CARCINOMA_VS_LOBULAR_NORMAL_DN	0.0012414	0.3058679	Cryab, Sfrp1, Zbtb16, Atp1a2, Atp10d, Mamdc2
GTPASE_ACTIVATOR_ACTIVITY	0.0012414	0.3058679	Arhgap4, Rgs16, Cdc42ep2, Gmip, Dock4, Rab3gap1
VARELA_ZMPSTE24_TARGETS_UP	0.0012487	0.3058679	Zbtb16, Rgs16, Mcart1, Pi4k2a, Slc20a1
BROWNE_HCMV_INFECTION_20HR_DN	0.0014108	0.3058679	Gsto1, Nr1d2, Fst, Nqo1, Pkig, F2r, Serpinh1, Svil
ASTON_MAJOR_DEPRESSIVE_DISORDER_DN	0.0014524	0.3058679	Cryab, Rbm8a, Atm, Cdc42ep1, Tmem87a, Gprc5b, Trf, Capn3, Pllp, Folh1
REACTOME_GAP_JUNCTION_TRAFFICKING	0.0015839	0.3058679	Gja1, Myo6, Gja5, Gjb1
RAS_GTPASE_BINDING	0.0015839	0.3058679	Ipo9, Cdc42ep2, Dock4, Rab3gap1
LIN_NPAS4_TARGETS_UP	0.0016129	0.3058679	Lpl, Mpp3, Abcf1, Usf1, Cdc42ep2, Mllt3, Prkaa2, Atp2c1, Pnma2
YAO_TEMPORAL_RESPONSE_TO_PROGESTERONE_CLUSTER_8	0.0021936	0.3813193	Usp14, Mthfd2, Zbtb16, Fst, Pdk3
HYDROLASE_ACTIVITY__ACTING_ON_CARBON_NITROGEN__BUT_NOT_PEPTIDE__BONDS	0.0021936	0.3813193	Nit1, Arg2, Mthfd2, Sirt2, Dctd
NGUYEN_NOTCH1_TARGETS_DN	0.0024829	0.3988758	Mthfd2, Ppp2r1b, Epha4, Fndc3a, Slc20a1, Serpinh1
THUM_SYSTOLIC_HEART_FAILURE_DN	0.0025606	0.3988758	Per2, Hey2, Mpp3, Slc25a36, Klf12, Zfp295, Cdc42ep2, Spag9, Stk35, Limch1, Tulp4
RODWELL_AGING_KIDNEY_NO_BLOOD_DN	0.0025814	0.3988758	Lpl, Mut, Pclo, Slc25a36, Ppp2r1b, Fbxo3, Prkaa2, Dbt
GAUSSMANN_MLL_AF4_FUSION_TARGETS_F_DN	0.0028524	0.4153637	Lpl, Tmem47, Rragd, Nnmt
HOSHIDA_LIVER_CANCER_SURVIVAL_DN	0.0028872	0.4153637	Usp14, Slc37a4, Tdo2, Ar, Ggcx, Dock4, Atp2c1, Gjb1
UEDA_PERIFERAL_CLOCK	0.0038006	0.4766850	Mesdc2, Nr1d2, Slc37a4, Per2, Glo1, Tsc22d1, F2r, Slc9a3r1, Lpin2
SCIBETTA_KDM5B_TARGETS_DN	0.0038998	0.4766850	Tubb5, Psip1,

			Ivns1abp, Epb4.1l1, Smc5, Limch1
WALLACE_PROSTATE_CANCER_RACE_DN	0.0038998	0.4766850	Pclo, Prkg2, Nqo1, Limch1, Adi1, Rab3gap1
FLOTHO_PEDIATRIC_ALL_THERAPY_RESPONSE_UP	0.0039126	0.4766850	Rps6, Sypl, Tmem87a, Rps9, Rpl17
SCHLOSSER_SERUM_RESPONSE_UP	0.0039764	0.4766850	Stxbp2, Map3k7, Klf12, Kcnb1, Ocel1, Tradd, Slc9a3r1, Ercc2
IWANAGA_CARCINOGENESIS_BY_KRAS_UP	0.0041643	0.4766850	Gja1, Slc15a2, Arg2, Adcy7, Rad23a, Ldhd, Supt7l, Cldn12, Sla2
POTTI_CYTOXAN_SENSITIVITY	0.0041671	0.4766850	Esd, Ggcx, Samd4, Rps9
REACTOME_GAP_JUNCTION_ASSEMBLY	0.0044689	0.4766850	Gja1, Gja5, Gjb1
RODWELL_AGING_KIDNEY_DN	0.0046427	0.4766850	Lpl, Mut, Pclo, Slc25a36, Fbxo3, Prkaa2, Dbt
BERENJENO_TRANSFORMED_BY_RHOA_FOREVER_DN	0.0046829	0.4766850	Gja1, Mtm1, Lrrfip1, F2r
I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	0.0049137	0.4766850	Gja1, Map3k7, Tradd, Atp2c1, F2r, Slc20a1, Rpl17
BHATTACHARYA_EMBRYONIC_STEM_CELL	0.0051300	0.4766850	Gja1, Tubb5, Psip1, Mthfd2, Cachd1, Serpinh1
POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	0.0051300	0.4766850	Gja1, Tradd, Atp2c1, F2r, Slc20a1, Rpl17
SMALL_GTPASE_BINDING	0.0052399	0.4766850	Ipo9, Cdc42ep2, Dock4, Rab3gap1
INOSITOL_OR_PHOSPHATIDYLINOSITOL_KINASE_ACTIVITY	0.0053410	0.4766850	Atm, Smg1, Pi4k2a
LE_EGR2_TARGETS_DN	0.0057983	0.4766850	Lpl, Zbtb16, Sirt2, Tmem40, Limch1, Efhd1, Gjb1
RICKMAN_TUMOR_DIFFERENTIATED_MODERATELY_VS_POORLY_UP	0.0058395	0.4766850	Ramp1, Eif2s2, Ipo9, Pop4
RICKMAN_TUMOR_DIFFERENTIATED_MODERATELY_VS_POORLY_DN	0.0058395	0.4766850	Ramp1, Eif2s2, Ipo9, Pop4
COLIN_PILOCYTIC_ASTROCYTOMA_VS_GLIOBLASTOMA_UP	0.0058395	0.4766850	Tnfrsf21, Gprc5b, Trf, Atp1a2
GTPASE_BINDING	0.0058395	0.4766850	Ipo9, Cdc42ep2, Dock4, Rab3gap1
YAGI_AML_FAB_MARKERS	0.0058853	0.4766850	Rab27b, Zbtb16, Timp2, Gp1ba, Ifi30, Pkig, Sorl1, F2r, Fam20b, Serpinh1
KLEIN_PRIMARY_EFFUSION_LYMPHOMA_DN	0.0059473	0.4766850	Fads3, Dbi, Sypl, Gatm, Pkig
STEARMAN_LUNG_CANCER_EARLY_VS_LATE_DN	0.0059473	0.4766850	Lpl, Myo7a, Gja5, Usf1, Mpeg1
NELSON_RESPONSE_TO_ANDROGEN_UP	0.0062236	0.4766850	Lman1, Gucy1a3,

			Dbi, Tsc22d1, Zbtb10, Inpp4b
BREDEMEYER_RAG_SIGNALING_NOT_VIA_ATM_DN	0.0064292	0.4766850	Slc15a2, Pdxdc1, Abhd14a, Jakmip1, Pi4k2a
LINDGREN_BLADDER_CANCER_CLUSTER_1_UP	0.0064508	0.4766850	Tmem19, Gsto1, Esd, Map3k7, Mllt3, Tm2d2, Ints10
LIU_COMMON_CANCER_GENES	0.0064831	0.4766850	Prdx2, Haus2, Lrrfip1, Dbt
FOURNIER_ACINAR_DEVELOPMENT_EARLY_DN	0.0065127	0.4766850	Amigo2, Slc20a1
REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE	0.0070410	0.5064699	Gja1, Tradd, Atp2c1, F2r, Slc20a1, Rpl17
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_UP	0.0078299	0.5516386	Rab27b, Il15, Fbxl20, Arg2, Fcer2a, Gatm, Inpp4b, Ifit3, Ccr6
CELL_CELL_ADHESION	0.0079334	0.5516386	Vangl2, Bmp1, Atp2c1, Cldn12, Amigo2, Cercam
GRAHAM_CML_DIVIDING_VS_NORMAL_QUIESCENT_DN	0.0084089	0.5628683	Cxcl13, Gucy1a3, Sorl1, Mllt3, Limch1, Svil
SMALL_GTPASE_REGULATOR_ACTIVITY	0.0086312	0.5628683	Arhgap4, Cdc42ep2, Gmip, Dock4, Rab3gap1
PROTEIN_N_TERMINUS_BINDING	0.0086910	0.5628683	Atm, Zwint, Sla2, Ercc2
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_16D_DN	0.0087372	0.5628683	Pi4k2b, Prdx2, Capn3, Dock4, Lrrfip1, Pyroxd2, Zcchc11
GARCIA_TARGETS_OF_FLI1_AND_DAX1_DN	0.0088085	0.5628683	Pldn, Slc7a11, Zwint, Ppcdc, Tprkb, Rg9mtd2, Haus2, Amdhd2
REACTOME_G2_M_TRANSITION	0.0089044	0.5628683	Actr1a, Tubb5, Wee1, Sfi1, Cep192, Haus2
GINESTIER_BREAST_CANCER_ZNF217_AMPLIFIED_UP	0.0092540	0.5758241	Itch, Pldn, Sp1, Slc25a36, F2r
KEGG_PYRUVATE_METABOLISM	0.0095235	0.5758241	Aldh7a1, Glo1, Ldhd, Me2
TRANSLATION_REGULATOR_ACTIVITY	0.0095235	0.5758241	Eif2s2, Gspt2, Abcf1, Samd4

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