IDENTIFICATION OF MOLECULAR MECHANISMS THAT UNDERLIE THE DEVELOPMENT AND TREATMENT OF MANIC-LIKE BEHAVIORS IN MICE: THE IMPORTANCE OF CHOLECYSTOKININ AND CHROMATIN MODIFICATIONS

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DEDICATION

I dedicate this work to my parents, Kathy and Tom Peck, to my grandparents Glen and Doris Arey, my siblings Olen, Julia, and Bianca, and to the rest of my family and friends.

"Such is oft the course of deeds that move the wheels of the world: small hands do them because they must, while the eyes of the great are elsewhere."

J.R.R. Tolkien

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by

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IDENTIFICATION OF MOLECULAR MECHANISMS THAT UNDERLIE THE DEVELOPMENT AND TREATMENT OF MANIC-LIKE BEHAVIORS IN MICE: THE IMPORTANCE OF CHOLECYSTOKININ AND CHROMATIN MODIFICATIONS

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Bipolar disorder (BPD) is a severe and chronic psychiatric disease that is defined by the occurrence of one or more manic or mixed episodes. The underlying cause of BPD, as well as the mechanisms of action of current pharmacological treatments for BPD, are poorly understood. Mice with a mutation in the *Clock* gene (*Clock*Δ19) have been identified as a model of mania that respond to lithium treatment; however, the mechanisms that underlie this phenotype, and the changes in the brain that are necessary for lithium's effectiveness on these mice remain unclear. Here we find that *Cholecystokinin* (*Cck*) is a direct transcriptional target of CLOCK and levels of *Cck* are reduced in the ventral tegmental area (VTA) of *Clock*Δ19 mice. Selective knock-down of *Cck* expression *via* RNA interference (RNAi) in the VTA of wild type mice is sufficient to produce a manic-like phenotype. Moreover, chronic treatment with lithium restores *Cck* expression to near wild type and this increase is necessary for the therapeutic actions

of lithium. The decrease in *Cck* expression in the *Clock*Δ19 mice is due to a lack of interaction with the histone methyltransferase, MLL1, resulting in decreased histone H3K4me3 and gene transcription, an effect reversed by lithium. We also found that another mood stabilizer, valproate (VPA), has mood stabilizing effects in the *Clock*Δ19 mice, and that VPA causes a restoration of *Cck* levels in the VTA, similar to lithium. Further investigation into the regulation of the *Cck* gene by VPA identified a role for histone acetylation in the therapeutic actions of mood stabilizers in the *Clock*Δ19 mice. Both lithium and VPA cause increases in histone acetylation at the *Cck* promoter, though potentially through different mechanisms. Lithium treatment may cause recruitment of specific chromatin remodeling complexes, while VPA appears to regulate the *Cck* gene through HDAC inhibition. The importance of HDAC inhibition in VPA's therapeutic actions was strengthened by the finding that administration of the specific Class I and IIb HDAC inhibitor suberoylanilide hydroxamic acid was sufficient to rescue manic-like behaviors in the *Clock*Δ19 mice. These studies identify a key role for *Cck* in the development and treatment of mania, and for regulation of chromatin modifications in the therapeutic actions of mood stabilizers.

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PRIOR PUBLICATIONS

Enwright III, J.F., Wald M., Paddock, M., Hoffman, E., **Arey R**., Edwards, S., Spencer, S., Nestler, E.J., McClung, C. 2010. DELTA FosB indirectly regulates *Cck* promoter activity. Brain Research. 1329:10-20

Arey R.N., Enwright J.F., Spencer S.M., Falcón E., Ozburn A.R., and McClung C.A. (Submitted) An Important Role for Cholecystokinin, a CLOCK Target, in the Development and Treatment of Manic-Like Behaviors

Arey R.N. and McClung C.A. (Submitted) An Inhibitor of Casein Kinase 1ε/δ Partially Normalizes the Manic-Like Behaviors of the *Clock*Δ19 Mouse

Spencer S.M., Sidor M.M., **Arey R.N.**, Jacobson J.P.R., Dzirasa K., Tye K.M., Enwright J.F., Caron M., Deisseroth K., and McClung C.A. (Submitted) Diurnal rhythms in dopamine synthesis and activity are important in mood and anxiety related behavior

Arey R.N., Spencer S.M., Ghose S., Tamminga C., and McClung C.A. (In preparation) Regulation of Histone Acetylation is Important for the Therapeutic Actions of Pharmacological Agents for the Treatment of Manic-Like Behaviors.

Falcón, E., Ozburn, A.R., Mukherjee, S., **Arey, R.N.**, Spencer, S.M., and McClung, C.A. (In preparation) Npas2 regulates cocaine reward and dopaminergic receptors in the ventral striatum.

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AAV – Adeno-Associated Virus

AcH3 – Acetylated Histone H3

AcH4 – Acetylated Histone H4

Akt – Protein Kinase B

AMPA – (2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid)

ANOVA– Analysis of Variance

ANK3 – Ankyrin3

ATP – Adenosine Triphosphate

β-TRCP – β–Transducin Repeat Containing Protein

BDNF – Brain-derived Neurotrophic Factor

bHLH-PAS – Basic helix-loop-helix-PAS

BMAL1 – Brain and Muscle ARNT-like protein 1

BPD – Bipolar Disorder

CACNA1C – Calcium Channel Subunit $Ca_v1.2$

CBP – CREB Binding Protein

CCK – Cholecystokinin

CCK_A – Cholecystokinin receptor A subtype

CCK_B – Cholecystokinin receptor B subtype

cDNA – Complementary DNA

ChIP – Chromatin Immunoprecipitation

CK1 – Casein Kinase 1

CLOCK – Circadian Locomotor Output Cycles Kaput

CRE – Cyclic AMP Response Element

CREB – Cyclic AMP Response Element Binding Protein

Cry – Cryptochrome

DA – Dopamine

DAG – Diacylglycerol

Darpp-32– Protein Phosphatase 1, regulatory subunit 1B

DBP – D-box Binding Protein

DISC1 – Disrupted in Schizophreina 1

DNA – Deoxyribonucleic Acid

DRD1– Dopamine D1 Receptor

DRD4 – Dopamine D4 Receptor

DSM-IV- Diagnostic and Statistical Manual of Mental Disorders IV

DTT - Dithithreitol

EGTA – Ethylene Glycol Tetraacetic Acid

ENU – *N*-ethyl-*N*-nitrosurea

EPAS1 – Endothelial PAS domain protein 1

EPM – Elevated Plus Maze

ERK – Extracellular Signal-Regulated Kinases

FST – Forced Swim Test

GABA – γ-Aminobutyric-Acid

Gapdh – Glyceraldehyde 3-Phosphate Dehydrogenase

GFP – Green Fluorescent Protein

GluR1 – AMPA Receptor Subunit 1

GSK-3β – Glycogen Synthase Kinase-3β

H3K4me3 – Trimethylated Histone H3 at Lysine 4

HAT – Histone Acetyltransferase

HDAC – Histone Deacetylase

HDACi – Histone Deacetylase Inhibitor

HIF1α – Hypoxia Inducible Factor 1α

HMT – Histone Methyl Transferase

HOP-β-CD – 2-hydroxypropyl-β-cyclodextrin

IMPase – Inositol Monophosphatase

IP_3 – 1,4,5-tris-phosphate

IPSRT – Interpersonal and Social Rhythm Therapy

L-DOPA – L-3,4-dihydroxyphenylalanine

LCM – Laser Capture Microdissection

LiCl – Lithium Chloride

MLL1 – Mixed lineage leukemia 1

MLL4 – Mixed lineage leukemia 4

mRNA – Messenger RNA

NAc – Nucleus Accumbens

NMDA – N-Methyl-D-Aspartic Acid

NPAS2 – Neuronal PAS domain protein 2

PC12 – Rat Pheochromocytoma Cell Line

PCR – Polymerase Chain Reaction

Per – Period

PIP_2 – Phosphatidylinositol 4,5-bisphosphate

PKC – Protein Kinase C

PP1– Protein Phosphatase 1

PP2A – Protein Phosphatase 2A

qPCR – Quantitative Polymerase Chain Reaction

RC – Regular Chow

REV-ERBA – Reverse Orientation Erb

RNA– Ribonucleic Acid

ROR – Retinoic Acid Receptor-Related Orphan Receptor

SAHA – Suberoylanilide Hydroxamic acid

SB – Sodium Butyrate

SCN – Suprachiasmatic Nucleus

shRNA – Short Hairpin Ribonucleic Acid

SIRT1 – Sirtuin 1

TH – Tyrosine Hydroxylase

TRE – TPA DNA Response Element

TSA – Trichostatin A

Vipr2 – Vasoactive Intestinal Peptide Receptor 2

VTA – Ventral Tegmental Area

VPA – Sodium Valproate/Valproic Acid

WT – Wild Type

CHAPTER ONE

INTRODUCTION

Bipolar disorder

Bipolar disorder (BPD) I is a psychiatric disorder defined by the American Psychiatric Association as the occurrence of one or more manic or mixed episode over the course of a lifetime (Miklowitz and Johnson 2006). In order to be classified as BPD I, this manic episode must be accompanied by hospitalization or last for a period of one week. Symptoms of mania include feelings of euphoria or irritability as well as hyperactivity, decreased need for sleep, grandiosity, racing thoughts and distractibility, increased talkativeness, and an increase in impulsive behavior and involvement in pleasurable and goal-directed activities (Miklowitz and Johnson 2006; Arora and Daughton 2007). In addition to manic episodes, patients with BPD also often experience periods of depression, which are characterized by dysphoria, prominent fatigue, feelings of worthlessness, anhedonia, and suicidal ideations (Miklowitz and Johnson 2006). In fact, BPD is classically referred to as "manic-depressive illness". Additional mood states are also experienced, including mixed episodes, which have characteristics of mania and depression, as well as euthymia, or normal mood. Other diseases in the BPD spectrum include BPD II, which is defined by the lifetime occurrence of a hypomanic episode, which is less severe and prolonged than a manic episode, and cyclothymia, in which a patient cycles between periods of some of the symptoms of hypomania and depression,

but do not fully meet the criteria for either major depression or hypomania (Miklowitz and Johnson 2006).

Approximately 1-3% of the United States population suffers from BPD, and it is the sixth leading cause of disability worldwide (Miklowitz and Johnson 2006). Both men and women are equally likely to develop the disease. The median age of onset is 25 years old, but 25% of patients begin to display symptoms by the age of 17, and generally have a poorer disease outcome, including rapid cycling in adulthood (Coryell, Solomon et al. 2003). Additionally, average life expectancy is lower for BPD patients when compared to the general population (Laursen 2011). Functional impairment is common in BPD, with only ~35% of patients being able to function at a level that existed prior to the previous episode (Zarate, Tohen et al. 2000; Sanchez-Moreno, Martinez-Aran et al. 2009). This functional impairment includes decreased capacity to work, live independently, and maintain normal social and familial relationships (Sanchez-Moreno, Martinez-Aran et al. 2009). In fact, only 33% of BPD patients can work full time, and over 50% are unable to work or can only work under direct supervision, which is higher than patients diagnosed with unipolar depression (Miklowitz and Johnson 2006; Sanchez-Moreno, Martinez-Aran et al. 2009).

In addition to this psychosocial impairment, there have been recent studies reporting cognitive impairments in BPD patients as well, though typically not as severe as those reported in schizophrenia (Zarate, Tohen et al. 2000; Sanchez-Moreno, Martinez-Aran et al. 2009). The most prominent form of impairment is that of verbal learning and memory and executive functions, which has also been commonly associated with work impairments (Martinez-Aran, Vieta et al. 2004; Balanza-Martinez, Selva et al.

2009). Functional impairments can also be worsened by the fact that BPD has a very high comorbidity rate. Anxiety disorders are common in BPD patients, with 18% of patients diagnosed with generalized anxiety disorder and 51% having at least one type of anxiety disorder (Simon 2009). Another prominent comorbidity in BPD is substance abuse disorders, with approximately 50-60% of patients reporting alcohol or substance abuse (Miklowitz and Johnson 2006; Balanza-Martinez, Selva et al. 2009). Alcohol is abused by about 40% of BPD patients and other substances are abused by approximately 15% of BPD patients. Substance abuse comorbidity is often associated with an early age of onset of BPD and more severe subtypes and symptoms of BPD (Levin and Hennessy 2004). The reason for this high comorbidity is unknown, though theories include attempts to "self-medicate" affective symptoms, or to enhance elevated mood in a manic or hypomanic state (Levin and Hennessy 2004). Comorbidity with general medical conditions in BPD patients also exceeds national averages, with the most prominent being problems such a hypertension, hyperlipidemia, type 2 diabetes, and obesity (Kilbourne 2005). These comorbidities possibly play a role in the reduced life expectancy of BPD patients, but suicide risk also contributes as well. Suicide risk includes suicidal ideation, nonfatal suicide attempts, and suicide. BPD has higher suicide risk than any other psychiatric disease, with as many as 50% of people with BPD attempting suicide over their lifetime, and an overall suicide rate of 10-15%, about 30-fold higher than the general population (Miklowitz and Johnson 2006; McIntyre, Muzina et al. 2008).

Because it is a complex psychiatric disease, the exact cause or causes of BPD have yet to be elucidated. There is clearly a genetic component to disease incidence as

there is a 57% concordance rate for identical twins, while it is only 14% for fraternal twins (Miklowitz and Johnson 2006). The disease is highly heritable, with children of parents with BPD four times more at risk than children of healthy parents to develop BPD, and three times more likely to develop a non-BPD psychiatric disease (McGuffin, Rijsdijk et al. 2003; Miklowitz and Johnson 2006). Linkage analysis studies have not identified any specific susceptibility genes for BPD, but linkage has been found on chromosome 6q and 8q (Smoller and Gardner-Schuster 2007). When subphenotypes in the disease were studied, there was linkage to 3q28, 18p, and 21q for age of onset (Smoller and Gardner-Schuster 2007). However, these linkage studies have been replaced by genome wide association studies, which have identified putative genes linked to BPD, including the calcium channel subunit $Ca_v1.2$ (*CACNA1C*) and ankyrin 3 (*ANK3*), which is involved in cell motility, activation, proliferation, contact, and the maintenance of membrane domains that modulate the activity of neuronal sodium channels as genes linked to BPD (Alsabban, Rivera et al. 2011). Association studies focused on candidate genes have also found genes that are involved in circadian rhythms, dopaminergic and serotinergic transmission, neural development and neurotrophic factors are associated with BPD (Escamilla and Zavala 2008; Serretti and Mandelli 2008). Interestingly, BPD seems to share some common genetic risk factors with schizophrenia including DISC1, and chromosome regions 18p11, 13q32, and 22q11 (Miklowitz and Johnson 2006; Porteous 2008).

There are many theories as to which neuronal processes and circuits are disrupted that can lead to BPD, including ion channel disruption, mitochondrial dysfunctions, and defects in calcium signaling (Stork and Renshaw 2005; Quiroz, Gray et al. 2008). It is

commonly thought that neurotransmitter dysregulation plays a role in the development of BPD. This dysregulation can also be partly responsible for altered cellular signaling, because neurotransmitters are responsible for the activation of specific cascades that have been linked to BPD. Various neurotransmitters are thought to play a role in bipolar disorder, including serotonin, acetylcholine, norepinephrine, and dopamine (Manji, Quiroz et al. 2003; Miklowitz and Johnson 2006). The present focus will be on the mesolimbic dopamine (DA) circuit and its possible role in the disease.

Dopamine is a member of the catecholamine family of neurotransmitters which also include epinephrine and norepinephrine. The source of DA in the mesolimbic DA circuit is ventral tegmental area (VTA). VTA dopaminergic neurons project to the nucleus accumbens (NAc), prefrontal cortex, and other limbic regions (Ikemoto 2007). The mesolimbic dopaminergic circuit is known to be involved in several behaviors, including motor control, cognition, as well as reward and motivation (Nestler 2005; Nestler and Carlezon 2006). The VTA-NAc circuit is known to regulate mood related behaviors, and has recently been implicated in depression (Krishnan and Nestler 2010). In reward signaling, VTA neurons release dopamine into the NAc whenever a rewarding stimulus is received. One model for dopaminergic involvement in BPD is that there are imbalances within dopaminergic transmission during the different phases of the disease. In the manic state, this circuit could be overactive, causing excess levels of dopamine production and release. To support this hypothesis, it has been demonstrated that enhancers of dopaminergic transmission, such as L-DOPA, amphetamine, cocaine and bromocriptine, can precipitate mania (Miklowitz and Johnson 2006; Brugue and Vieta 2007). Depletion of tyrosine, a dopamine precursor, in the diet of patients with bipolar

disorder, decreases the severity of the manic phase (Brugue and Vieta 2007). Furthermore, dopamine's role in motor control implicates it in the disease, because increases in locomotion are one of the defining characteristics of the manic phase of the disease (Miklowitz and Johnson 2006). In addition, the depressive phase could be linked to a deficiency of dopamine, for lack of motivation and anhedonia are prominent features of bipolar depression (Miklowitz and Johnson 2006). Moreover, recent human genetic studies have found various components of dopaminergic transmission to be linked to BPD, including the dopamine D1 and D4 receptors (*DRD1, DRD4*), and the dopamine transporter (Escamilla and Zavala 2008).

It is difficult to completely model a complex disease such as BPD in organisms such as mice or rats; instead, specific aspects of the disease are often studied. In wild type (WT) animals, pharmacological manipulations can be employed. One of the most common pharmacological manipulations is psychostimulant induced hyperactivity, in which a mouse or rat becomes sensitized to repeated injections of amphetamine, methamphetamine, or cocaine to model the hyperactivity seen in mania (Kato, Kubota et al. 2007; Martinowich, Schloesser et al. 2009; Nestler and Hyman 2010). This method has some predictive validity, as treatment with the mood stabilizers lithium or valproate can blunt an animal's response to a psychostimulant, and thus may be useful for initial screening of compounds to treat mania (Kato, Kubota et al. 2007; Martinowich, Schloesser et al. 2009; Nestler and Hyman 2010). Another manipulation that is used to mimic mania in WT animals is sleep deprivation, which is chosen in part because sleep deprivation can precipitate manic episodes (Kato, Kubota et al. 2007). Sleep deprivation can model several manic-like behaviors including hyperactivity, increased aggression,

and hypersexuality (Kato, Kubota et al. 2007; Martinowich, Schloesser et al. 2009). However, a major weakness in these models is that molecular and cellular changes in the brain following these paradigms in WT animals may not be the same as those found in mania, which is a pre-existing disease state (Nestler and Hyman 2010). More recently, genetically manipulated mice have been used to model BPD, most often the manic phase. Behaviors that can be modeled in mice include hyperactivity, increase in goal-directed behavior and substance abuse, excessive involvement in pleasurable activities with potential for painful consequences (or increased risk taking), and elevated mood. These can be measured by assessing locomotor activity, preference for both natural and drug induced reward, decreases in anxiety related behavior, which may correlate with increased risk taking, and decreases in depression related behavior (Chen, Henter et al. 2010). Using these behaviors, potential pathways that are involved in the development of manic-like behaviors have been identified. These include glutamatergic and dopaminergic neurotransmission, GSK-3β and ERK signaling, and circadian rhythms (Martinowich, Schloesser et al. 2009; Le-Niculescu, Patel et al. 2010; Coque, Mukherjee et al. 2011).

Circadian Rhythms

Circadian rhythms are daily cycles in behavior and physiology that are coordinated to the 24 hour day by various stimuli known as zeitgebers ("time-givers"), with the daily light-dark cycle being the most potent for mammals (Ko and Takahashi 2006). The sleep/wake cycle is the most prominent function controlled by circadian

rhythms, but other processes regulated by circadian rhythms include feeding behavior, hormone secretion, body temperature, blood pressure, and metabolism (Takahashi, Hong et al. 2008). These rhythms are sustained in the absence of environmental cues, indicating that they are generated by an "internal clock" (Takahashi, Hong et al. 2008; Lowrey and Takahashi 2011). The central pacemaker that controls these rhythms is the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which is entrained by light inputs from the retina via the retinohypothalamic tract (Reppert and Weaver 2001). The SCN acts to coordinate oscillators found in other brain regions and peripheral organs via direct neural connections and hormonal secretion (Reppert and Weaver 2001; Lowrey and Takahashi 2011) .

Within the SCN and other oscillator organs, circadian rhythms are controlled by a signaling network known as the "molecular clock." This molecular clock is comprised of a series of transcriptional-translational feedback loops that cycle over the course of twenty-four hours (Ko and Takahashi 2006; Takahashi, Hong et al. 2008). The main transcriptional activators of this loop are the bHLH-PAS transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1), which form a heterodimer and bind to the E-box (5'-CANNTG-3') or E'box (5'-CACGTT-3') site of promoters of the *Period (Per)* and *Cryptochrome (Cry)* genes, along with other clock controlled genes, and enhance their transcription (Ko and Takahashi 2006; Takahashi, Hong et al. 2008). PER and CRY provide negative feedback after they heterodimerize and form a complex with other proteins and re-enter the nucleus. In the nucleus, this PER/CRY containing complex accumulates and directly

inhibits the activity of CLOCK/BMAL1, thus inhibiting their own transcriptional activation (Lowrey and Takahashi 2011).

There are additional layers of regulation of molecular clockwork. In the forebrain, there is a CLOCK analogue neuronal PAS domain protein 2 (NPAS2), that heterodimerizes with BMAL1 to control gene expression in a similar manner to CLOCK (Reick, Garcia et al. 2001). It also appears that NPAS2 can functionally replace CLOCK in other regions in the absence of a CLOCK protein (DeBruyne, Weaver et al. 2007). An adjoining loop of the molecular clock involves transcriptional regulation of the *Bmal1* gene. This loop is regulated by the retinoic acid receptor-related orphan receptor (ROR) family members RORα, RORβ, and RORγ, which act as positive regulators of the *Bmal1* gene, and the reverse orientation erbA (REV-ERB) nuclear receptors REV-ERBα and REV-ERBβ negatively regulate *Bmal1* expression (Reppert and Weaver 2001; Ko and Takahashi 2006; Takahashi, Hong et al. 2008).

Furthermore, posttranslational modifications to PER and CRY, can alter their stability, dimerization, and nuclear entry, and thus their ability to regulate CLOCK/BMAL1 (Harms, Young et al. 2003; Ko and Takahashi 2006). Phosphorylation of PER by casein kinase 1 ε and δ (CK1ε/δ) regulates PER subcellular localization and ability to inhibit CLOCK/BMAL1. Phosphorylation CK1ε/δ also targets PER for ubiquitin-dependent degradation by the 26s proteasome (Lowrey and Takahashi 2011). Another kinase that regulates circadian rhythms is glycogen synthase kinase-3β (GSK-3β), which is involved in many intracellular signaling pathways (Doble and Woodgett 2003; Lowrey and Takahashi 2011). Phosphorylation of PER2 by GSK-3β promotes its nuclear localization, while phosphorylation of CRY2 leads to degradation (Harada, Sakai

et al. 2005; Iitaka, Miyazaki et al. 2005). GSK-3β can also affect CLOCK and BMAL1. This can occur both directly, as GSK-3β can phosphorylate both of these proteins and promote their degradation, or indirectly, as studies have found that GSK-3β mediated phosphorylation stabilizes REV-ERBα, leading to repression of *Bmal1* transcription (Yin, Wang et al. 2006; Spengler, Kuropatwinski et al. 2009; Sahar, Zocchi et al. 2010). A schematic diagram depicting the roles of the molecular clockwork is shown in Figure 1-1.

In addition to the transcriptional-translational feedback loop, it has been found that there is an important role for epigenetics in the regulation of circadian rhythms. Epigenetics refers to changes in gene expression that are caused by mechanisms that are not due to differences in DNA sequence (Zhang and Meaney 2010). One of the most common epigenetic mechanisms is altering post-translational modifications on the tails of histone proteins, which are octomeric protein complexes that DNA is wrapped around to form chromatin (Zhang and Meaney 2010). These modifications can regulate how easily transcription factors can access DNA, and thus regulate gene expression (Zhang and Meaney 2010).

These best studied of these modifications is acetylation of lysine (K) residues, which is associated with gene activation, and deacetylation, which is associated with inactivation of a gene (Zhang and Meaney 2010). This modification is regulated by histone acetyltransferases (HAT), which add acetyl groups to lysines, and histone deacetylases (HDACs), which remove acetyl groups, and are though to be transcriptional silencers (Zhang and Meaney 2010). HDACs can be divided into 4 classes of zinc-dependent HDACs- Class I (HDAC1, 2, 3, 8), Class IIa (HDAC4, 5, 7, 9), Class IIb (HDAC 6, 10), and Class IV (HDAC11), and the Class III HDACs, or sirtuins, which are

modulated by the cofactor NAD^+ (Bantscheff, Hopf et al. 2011). Class I HDACs remain in the nucleus, whiles Class IIa shuttle between the nucleus and the cytoplasm. Class IIb HDACs do not function epigenetically, but instead regulate protein folding and turnover, and play a role in microtubule dynamics (Bantscheff, Hopf et al. 2011). Other common histone modifications include methylation, which is regulated by histone methyltransferases (HMTs) and histone demethylases. Methylation can be activating or repressing depending upon the site of the modification (Masri and Sassone-Corsi 2010). Histones can also be phosphorylated, which is typically associated with activation (Masri and Sassone-Corsi 2010).

Circadian oscillation of levels of various post-translational modifications to histone tails have been found, including histone H3 acetylation (AcH3), phosphorylation of histone H3 at ser10, and methylation at histone H3 lysine4 (H3K4me) (Etchegaray, Lee et al. 2003; Masri and Sassone-Corsi 2010). Interestingly, the CLOCK/BMAL1 complex has been found to have intrinsic HAT activity, and can regulate levels of histone acetylation at H3K9/K14, which may generate a permissive state that allows other HATs, such as CBP/p300 to be recruited to clock controlled genes (Doi, Hirayama et al. 2006; Masri and Sassone-Corsi 2010). CLOCK/BMAL1 also interact with the Class III HDAC SIRT1 (Nakahata, Kaluzova et al. 2008). SIRT1, when complexed with CLOCK/BMAL1, was found to regulate rhythms in histone acetylation at H3K9/K14 (Nakahata, Kaluzova et al. 2008). Other HDACs may be involved in the circadian epigenome, as expression of HDAC1 and HDAC2 oscillates in a circadian manner, and HDAC3 has been found to regulate the expression of peripheral circadian genes (Masri and Sassone-Corsi 2010). Recently, CLOCK/BMAL has been found to associate with

the histone methyltransferase MLL1, which is critical for oscillation in H3K4me3, and activation of circadian genes (Figure 1-2).

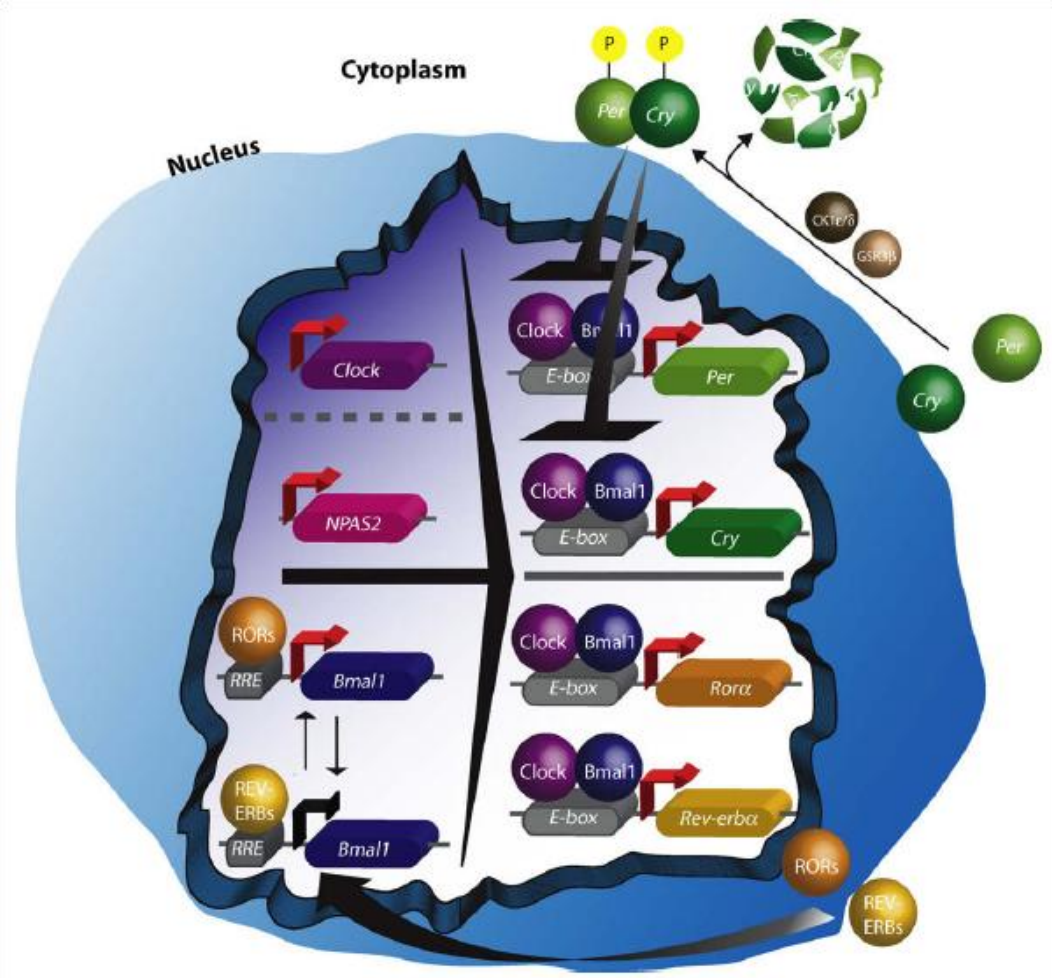


Figure 1-1. The molecular clockwork. Schematic diagram depicting the transcriptional-translational feedback look that controls circadian rhythms. CLOCK and BMAL1 heterodimerize and activate the transcription of *Per* and *Cry*. Over the course of 24 hours PER/CRY dimerize and enter the nucleus and inhibit CLOCK/BMAL1. PER/CRY can be regulated by phosphorylation via the kinases CK1ε/δ and GSK-3β. REV-ERBs and RORs form an adjoining loop that regulates *Bmal1* transcription. Adapted from (Falcon and McClung 2009).

It is critical to note that components of the molecular clockwork are found not only in oscillator regions, but throughout the brain and the body, where they may mediate other processes and physiological functions.

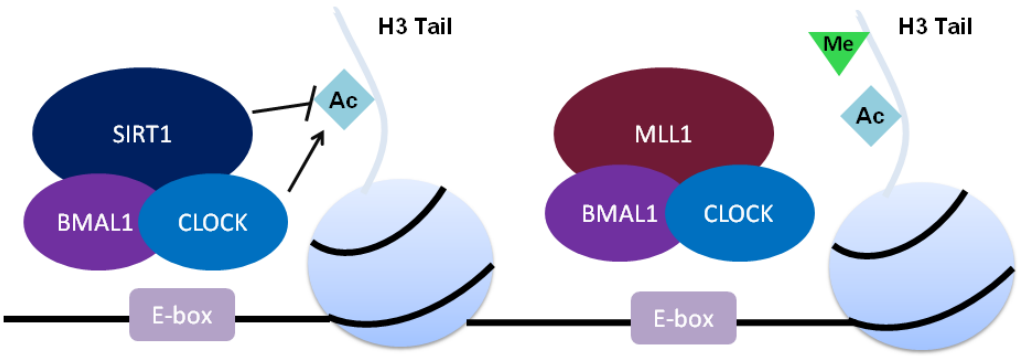


Figure 1-2. Regulation of chromatin modifications by CLOCK/BMAL1. Schematic diagram depicting regulation of the circadian epigenome by CLOCK/BMAL1. CLOCK promotes acetylation of histone H3 via its intrinsic HAT activity. The HDAC SIRT1 associates with CLOCK/BMAL1 and also regulates levels of H3 acetylation. CLOCK/BMAL1 also regulate circadian gene activation by forming a complex with MLL1. MLL1 methylates histone H3K4, which promotes gene transcription.

Disruptions in Circadian Rhythms are Associated with Bipolar Disorder

There is a growing body of evidence that links disruptions in circadian rhythms to BPD. Human genetic studies have found that the CLOCK gene is associated with BPD, and a 3111T/C polymorphisms in the CLOCK gene is linked to sleep disturbances in BPD and recurrence of bipolar depression in BPD (Serretti, Benedetti et al. 2003;

Benedetti, Dallaspezia et al. 2007; Soria, Martinez-Amoros et al. 2010). Additionally, polymorphisms in BMAL1, NPAS2, PER3, CRY2, and CK1, are associated with BPD (Mansour, Wood et al. 2006; Nievergelt, Kripke et al. 2006; Kripke, Nievergelt et al. 2009; Mansour, Talkowski et al. 2009; Sjoholm, Backlund et al. 2010).

In addition to human genetic evidence, various mouse models of BPD have alterations in genes linked to circadian rhythms. Mice bearing a point mutation in the *Clock* gene were generated by an ENU mutagenesis screen to identify molecules involved in circadian rhythms (Vitaterna, King et al. 1994). This point mutation causes a deletion of exon 19 in the CLOCK protein (CLOCKΔ19), which contains the transactivation domain, resulting in a dominant negative CLOCK function. CLOCKΔ19 can heterodimerize with BMAL1 and bind to E-box elements, but can no longer activate transcription. Behavioral characterization of the *Clock*Δ19 mice revealed a fairly complete manic-like phenotype. In addition to disrupted circadian rhythms, *Clock*Δ19 mice have a decreased need for sleep and are hyperactive, similar to BPD patients in the manic phase (Naylor, Bergmann et al. 2000; McClung, Sidiropoulou et al. 2005). The *Clock*Δ19 mice also display a number of manic-like mood related phenotypes, including decreased anxiety-related and depression-related behavior, which correlates with the increase in risk-taking behavior and elevated mood observed in human mania, respectively (Roybal, Theobold et al. 2007). In addition, *Clock*Δ19 mice have an increased preference for cocaine, sucrose, and intracranial self stimulation, indicating a hyperhedonic phenotype (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007). This is compelling because of the comorbidity with substance abuse in BPD (Strakowski and DelBello 2000). Furthermore, treatment with the mood-stabilizer

lithium reverses the mood and anxiety-related behavior of *Clock*Δ19 mice (Roybal, Theobold et al. 2007).

Transgenic mice overexpressing GSK-3β, a kinase that can regulate circadian rhythms, also display some manic-like behaviors, including hyperlocomotion and reduced immobility in the forced swim test (Prickaerts, Moechars et al. 2006). *Per2* knockout mice (*Per2*^{Brdm1}) also display decreased immobility and an increase in preference for alcohol (Spanagel, Pendyala et al. 2005; Hampp, Ripperger et al. 2008). In addition, mice lacking D-box binding protein (DBP), a transcriptional activator that is also known to be involved in circadian rhythms, have also been used to model both the manic and depressive phases of BPD (Le-Niculescu, McFarland et al. 2008). When compared to WT controls, DBP knockout animals display increased depression related behavior (Le-Niculescu, McFarland et al. 2008). Following exposure to both acute and chronic stress, they are hyperactive and show increased alcohol intake (Le-Niculescu, McFarland et al. 2008). Recently, our lab has also performed behavioral analysis on mice lacking BMAL1 (*Bmal1-/-).* Interestingly *Bmal1-/-* mice resemble *Clock*Δ19 mice, displaying both decreased anxiety and depression-related behavior (unpublished observations).

In addition to this genetic evidence, there is a great deal of clinical evidence linking circadian rhythms with BPD. Sleep disturbances, both insomnia and hypersomnia, are common in BPD and often precipitate manic or depressive episodes (Lamont, Legault-Coutu et al. 2007; Harvey 2011) , and disrupted sleep/wake cycles are part of the DSM-IV diagnostic criteria for both manic (decreased need for sleep) and depressive (insomnia or hypersomnia) episodes of BPD (Harvey 2011). Additionally,

bipolar patients have abnormal rhythms in physiological functions such as body temperature, blood pressure, and hormone secretion (McClung 2007). These observations contribute to the "social zeitgeber theory" for the course of BPD (Hlastala 2003). This theory suggests that BPD patients have "vulnerable" circadian clocks, as evidenced by their disrupted rhythms, and that stressful life events act to disrupt social zeitgebers, such as social interactions, meal times, and usual work hours (Hlastala 2003). These disruptions in turn disrupt circadian rhythms and lead to affective episodes (Hlastala 2003). To support this, Malkoff-Schwartz et al., found that events that disrupt social rhythms, which include sleep/wake cycle, work and social obligations, are reported more often in the time leading to a manic episode (Malkoff-Schwartz, Frank et al. 1998; Malkoff-Schwartz, Frank et al. 2000) .

Additionally, treatments for BPD may also affect circadian rhythms. The mood stabilizer lithium has been shown to cause a strong phase delay in circadian rhythms in many organisms, including humans (Klemfuss 1992). Early clinical reports suggest that this circadian effect plays a role in lithium's therapeutic actions. In a small number of patients, lithium ameliorated symptoms of BPD patients with abnormally short, but not long, circadian periods (Atkinson, Kripke et al. 1975; Kripke, Mullaney et al. 1978). A non-pharmacological form of treatment, Interpersonal and Social Rhythm Therapy (IPSRT), may act in part through stabilizing rhythms. IPSRT was developed with the social zeitgeber theory of bipolar disorder in mind, and sets out to aid patients in setting up regular daily routines to decrease the risk of disrupting their vulnerable circadian systems (Frank, Swartz et al. 2000). Under IPSRT, patients have very strictly controlled sleep/wake, social, and work schedules (Frank, Swartz et al. 2000). This treatment

approach has been found to effectively aid in mood stabilization in conjunction with pharmacological treatment (Frank, Kupfer et al. 2005).

Mood stabilizers for the treatment of Bipolar Disorder

Because bipolar disorder has several different mood states, there are three symptom targets for pharmacological treatment, manic states, mixed episodes, and depression. In the treatment of mania and mixed states, mood stabilizers are often most effective. As their name suggests, mood stabilizers ameliorate manic symptoms without triggering a depressive episode (Miklowitz and Johnson 2006). Mood stabilizers are not as efficacious in the treatment of bipolar depression, which can be ameliorated by standard antidepressant treatment; however, this treatment can often induce mania or accelerate mood cycling (Altshuler, Post et al. 1995; Miklowitz and Johnson 2006). Combining antidepressants with mood stabilizer treatment may help prevent the occurrence of a manic episode (Miklowitz and Johnson 2006). More recently, the anticonvulsant lamotrigine has been shown to be effective in the treatment of bipolar depression; however, it has risk of serious side effects (Miklowitz and Johnson 2006). The actions of mood stabilizers will be described in more detail below.

Lithium

Lithium has been used as a mood stabilizer for over 60 years and is seen as a first line drug in the treatment of BPD. Several studies have found that 40-80% of BPD patients show a remission of manic symptoms within two-three weeks of beginning lithium treatment (Vieta and Sanchez-Moreno 2008). In addition to being an effective

treatment for acute episodes in BPD, lithium can be used in the maintenance of the disease. Lithium administration can reduce the chance of a manic episode recurring by 50% (Vieta and Sanchez-Moreno 2008). It has been found to be more effective in preventing the recurrence of mania than depression; however, lithium appears to have antisuicidal effects as well (Baldessarini, Tondo et al. 2006). Lithium can also be used when combination therapy is necessary, as it is well tolerated when administered with other drugs such as anticonvulsants and antipsychotics (Freeman and Stoll 1998). There are several problems that can occur with lithium treatment. Numerous adverse side effects can be experienced including sedation, stomach irritation, thirst, motor tremors, weight gain, hypothyroidism, and kidney clearance issues (Miklowitz and Johnson 2006; Vieta and Sanchez-Moreno 2008). Because of these side effects and lithium's relatively narrow therapeutic index, nonadherance is often a problem, which carries a high risk for a "rebound manic" episode (Vieta and Sanchez-Moreno 2008).

Despite its use for decades, the mechanism by which lithium exerts its mood stabilizing effects is unknown. The drug has been studied extensively *in vitro* and in pre-clinical models; however, these studies have mostly been carried out in WT animals, because lithium can prevent psychostimulant induced hyperlocomotion. A drawback to these studies is that a WT animal better models a healthy control than a disease state, such as BPD. In these preclinical models, and *in vitro*, several potential mechanisms of action for lithium have been proposed.

At the level of neurotransmission, lithium has been found to affect neurotransmitters that have been linked to BPD. Early studies found that lithium inhibits depolarization-induced and calcium-dependent release of dopamine (Baldessarini and

Vogt 1988). Recent studies in our own lab have found that lithium can also reduce dopaminergic transmission in the *Clock*Δ19 mouse (Coque, Mukherjee et al. 2011). In addition it may stimulate the release of serotonin (Treiser, Cascio et al. 1981). These effects are likely mediated through the effects of lithium on various intracellular signaling cascades.

One of the most popular models for lithium's mechanism of action involves inhibition of the serine-threonine kinase glycogen synthase kinase - 3β (GSK-3β (Gould, Chen et al. 2004; Gould, Quiroz et al. 2004; Quiroz, Singh et al. 2004). Lithium inhibits GSK-3β by competing with magnesium, which is an important co-factor for the enzyme (Klein and Melton 1996; Ryves and Harwood 2001). This inhibition also renders GSK-3β unable to activate protein-phosphatase 1, which removes inhibitory phosphorylation of GSK-3β at serine 9 (Zhang, Phiel et al. 2003). This further reduces levels of active GSK-3β in the cell. GSK-3β functions as an intermediary in many cellular signaling pathways (Frame and Cohen 2001; Doble and Woodgett 2003) . It has over 30 identified targets within the cell, and can therefore play a role in many processes, including regulation of neuroprotection and apoptosis, circadian rhythms, neurite outgrowth, and cytoskeletal remodeling (Frame and Cohen 2001; Doble and Woodgett 2003; Iitaka, Miyazaki et al. 2005). In fact, lithium's ability to impact circadian rhythms had long been thought to be due to inhibition of GSK-3β, though this has recently become controversial (Iitaka, Miyazaki et al. 2005; Hirota, Lewis et al. 2008). Many of the targets of GSK-3β are transcriptional regulators including CREB, β-catenin, Myc, and c-Jun. Lithium can hypothetically regulate genes that these transcription factors target, and

the processes that they are involved in (Bullock and Habener 1998; Frame and Cohen 2001; Grimes and Jope 2001; Jope and Bijur 2002).

Recently, lithium has been found to also indirectly affect GSK-3β and G-proteins, specifically dopamine receptor D_2, signaling though regulation of a β-arrestin signaling complex (Beaulieu and Caron 2008; Beaulieu, Marion et al. 2008). β-arrestins can mediate G-protein signals by acting as scaffolds for protein complexes. The complex that lithium regulates consists of Akt, β-arrestin2, and protein phosphatase 2A (PP2A), and is downstream of D_2 receptors (Beaulieu and Caron 2008). This complex regulates the phosphorylation state of Akt, deactivating it. Akt normally phosphorylates GSK-3β in an inhibitory manner. Therefore, this complex usually promotes GSK-3β activity. Lithium was found to interefere with the formation of this complex, increasing inhibitory phosphorylation of GSK-3β, potentially in a magnesium dependent manner (Beaulieu and Caron 2008; Beaulieu, Marion et al. 2008).

Because lithium acts by competing with magnesium, it can also affect many other enzymes in the cell. Another potential mechanism of lithium's action that has been hypothesized is that of inositol depletion (Gould, Quiroz et al. 2004; Quiroz, Gould et al. 2004; Harwood 2005). In this model, lithium inhibits two different phosphatases, inositol polyphosphate 1-polyphosphatase and inositol monophosphatase (Harwood 2005) Both of these enzymes are involved in the recycling of myo-inositol, thus lithium treatment results in a depletion of myo-inositol levels within the cell. This in turn can affect levels of PIP_2, which is normally converted to the second messengers inositol 1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG) by phospholipase C. Altering IP_3/DAG signaling can affect many proteins, specifically protein kinase C (PKC) and

Calmodulin-dependent protein kinase II, and intracellular calcium signaling (Gould, Quiroz et al. 2004; Harwood 2005; Schloesser, Huang et al. 2008)

Valproate

Another mood stabilizer that is commonly used to treat BPD is valproate (VPA). VPA has other clinical uses, specifically in the treatment of epilepsy (Mitchell and Malhi 2002). It is similar to lithium in reducing manic symptoms in BPD patients, but it does have a faster onset of action than lithium, with clinical improvements occurring in approximately one week, as opposed to the two to three weeks for lithium treatment (Vieta and Sanchez-Moreno 2008). Little is known about the effectiveness of VPA in the treatment of bipolar depression (Mitchell and Malhi 2002). An advantage of VPA treatment is that it has a milder side effect profile than lithium. VPA side effects include nausea, weight gain, stomach pain, fatigue, depressed platelet counts, and elevated liver enzymes (Miklowitz and Johnson 2006; Vieta and Sanchez-Moreno 2008).

Similar to lithium, VPA is a small molecule that can affect a number of cellular signaling pathways. Because it has antiepileptic properties in addition to being effective as a mood stabilizer, VPA has been shown to affect GABAergic neurotransmission, specifically by increasing GABA levels in the brain (Gurvich and Klein 2002). This may be due to a decrease in the breakdown of GABA or by an increase in the conversion of glutamic acid to GABA, though it remains unclear which of the mechanisms causes this increase, or if it is a combination of the two (Gurvich and Klein 2002). VPA has also been shown to affect excitatory neurotransmission. VPA can inhibit voltage dependent Na^+ channels, and has been shown to reduce NMDA receptor dependent action potentials

and inhibit the binding of AMPA to its receptor (Gurvich and Klein 2002). VPA also can affect chromatin structure, as it has more recently been found to be a direct HDAC inhibitor (HDACi, (Phiel, Zhang et al. 2001). VPA is a fairly non-specific HDACi, affecting both Class I and II HDACs; therefore, it can potentially affect the expression of many genes through this mechanism (Phiel, Zhang et al. 2001; Gurvich and Klein 2002).

VPA also can affect some of the same cell signaling pathways as lithium treatment. There is controversy as to whether or not GSK-3β is a target of VPA, as some studies have found that VPA inhibits GSK-3β, while others have found that VPA does not directly inhibit GSK-3β activity (Chen, Huang et al. 1999; Phiel, Zhang et al. 2001; Jin, Kovacs et al. 2005). Though it is uncertain whether or not VPA can inhibit GSK-3β like lithium, it has been shown to affect some of the downstream targets of GSK-3β in a manner similar to lithium, including enhancement of β-catenin dependent transcription (Gurvich and Klein 2002). VPA has also been shown to reduce inositol signaling, though it affects *de novo* inositol biosynthesis and not inositol recycling, like lithium (Terbach and Williams 2009). Interestingly, VPA has also been shown to lengthen the circadian rhythms of *Drosophila*, though the effect is more modest than lithium treatment (Dokucu, Yu et al. 2005).

Other Treatments for Bipolar Disorder

Though lithium and VPA are two of the most commonly used drugs to treat BPD, there is an expanding range of drugs that have been employed in the treatment of this disease. Another anticonvulsant, carbamazepine, has been found to be effective in the treatment of mania and the anticonvulsant lamotrigine can treat bipolar depression

(Vieta and Sanchez-Moreno 2008). Traditional antipsychotic agents such as chlorpromazine and haloperidol as well as atypical antipsychotics like risperidone and olanzapine can also treat manic symptoms, often in conjunction with mood stabilizers like VPA or lithium (Vieta and Sanchez-Moreno 2008). Traditional antipsychotics block dopamine D_2 receptor function, while atypical antipsychotics block dopamine receptors as well as some serotonin receptors (Miklowitz and Johnson 2006).

Potential Common Targets of Mood Stabilizers

The actions of drugs for the treatment of BPD, more specifically mania, are numerous and diverse. They can affect many signaling pathways that can affect neuronal processes and function (Summarized in Table 1-1). It is therefore unlikely that one single protein is the target of all mood stabilizing drugs, but instead their various actions can affect targets, such as certain genes, through diverse mechanisms.

Because current treatments for BPD have many side effects and are not effective for all patients, there is a need for new therapies for the treatment of the disease. Studies in appropriate animal models are the best approach to identify potential drug targets. The *Clock*Δ19 mice are a particularly excellent model for these studies, because they have a behavioral phenotype that is strikingly similar to human mania, and respond to the mood stabilizer lithium. In the following chapters, the *Clock*Δ19 mice will be used to identify molecules involved in the development of manic-like behaviors and mechanisms by which mood stabilizing agents act with the hope of creating newer, more effective treatments for BPD.

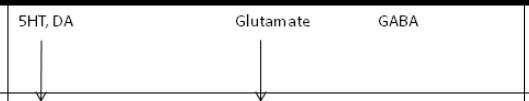
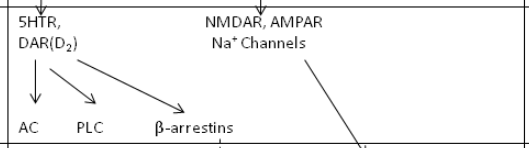
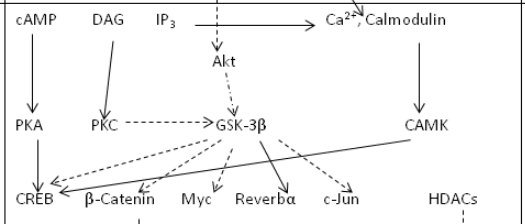
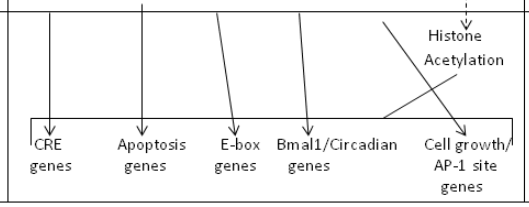
Level	Action	Pathways	Targets for available drugs?
Presynaptic Terminal	NT Release		Lithium, VPA, Anticonvulsants
Post-synaptic Terminal Inner Cell Membrane	NT receptor Activation of G proteins		Antipsychotics, VPA Lithium –β-arrestin complex formation;may modulate ACs
2^{nd} messengers Protein Kinases Regulatory proteins (TFs, HDACs)	Release of 2^{nd} messengers Protein Kinase activation Phosphorylation, Direct inhibition		Li, VPA- Inositol Depletion Li, VPA?- GSK-3β inhibition VPA- HDAC inhibition
DNA Chromatin	Chromatin Structure Regulation of Gene expression		

Table 1-1. Summary of drug actions for the treatment of BPD. A simplified diagram of the various cell signaling pathways regulated by mood-stabilizing medications. Full lines indicate stimulatoryand dashed lines indicate inhibitory effects. Many of these pathways converge on regulatory proteins and transcription factors that in turn can affect a number of target genes. List of Abbreviations: NT- neurotransmitter, 5HT- serotonin; DA- Dopamine, R-Receptor, AC- Adenylate cyclase, PLC- Phospholipase C, PKA- Protein Kinase A, PKC-Protein Kinase C, CREB- cAMP response element binding protein, CRE- cAMP response element, AP-1-activator protein 1.

CHAPTER TWO

AN IMPORTANT ROLE FOR CHOLECYSTOKININ, A CLOCK TARGET GENE, IN THE DEVELOPMENT AND TREATMENT OF MANIC-LIKE BEHAVIORS

Introduction:

Bipolar disorder (BPD) is a severe and chronic psychiatric disease that afflicts approximates 1-3% of the United States population (Kessler, Berglund et al. 2005). According to the DSM-IV, BPD is defined by the occurrence of one or more manic or mixed episodes. Though mania is the defining characteristic of BPD, it was once known as manic-depressive illness due to the multiple mood states that can be experiences as a result of this disease. Symptoms of mania include elevated mood, irritability, decreased need for sleep, hyperactivity, and an increase in risk taking behavior (Miklowitz and Johnson 2006). The depressive phase of BPD resembles major depressive disorder, with dysphoria, anhedonia, and disrupted sleep patterns. Mixed states, which have characteristics of mania and depression, as well as periods of euthymia, or normal mood, can also be experienced (Miklowitz and Johnson 2006). The underlying cause of BPD is unknown, though there is a growing body of evidence linking disruptions in circadian rhythms with the disease.

Circadian rhythms are approximately 24 hours cycles in biochemistry, physiology, and behaviors. External stimuli known as zeitgebers (time-givers) synchronize these rhythms to the day. The primary zeitgeber is the daily light-dark cycle

provided by the sun; however, other factors, such as food and drugs, can act as zeitgebers as well. The 'master' regulator of circadian rhythms is the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN acts to coordinate circadian rhythms in the brain and in other "slave oscillators," such as the liver, lungs, and kidneys (Takahashi, Hong et al. 2008). Within the SCN and other oscillator regions, circadian rhythms are controlled by the molecular clockwork, which is comprised of a series of autoregulatory transcriptional-translational feedback loops. The basic helix-loop-helix PAS transcription factors CLOCK and BMAL1 heterodimerize and activate transcription via the E-box (5'-CANNTG-3') or E'-box (5'-CACGTT-3') elements of target genes, including *Per* and *Cry*. PER and CRY heterodimerize and translocate into the nucleus, directly inhibiting the CLOCK/BMAL1 complex, and thus, their own transcription. The degradation of the PER/CRY complex relieves the inhibition of CLOCK/BMAL1 and re-initiates the transcriptional-translational feedback loop.

Recent human genetic studies have linked elements of the molecular clockwork to BPD. Polymorphisms in CLOCK and other circadian genes have been found to be associated with various aspects of bipolar disorder (Serretti, Benedetti et al. 2003; Mansour, Wood et al. 2006; Nievergelt, Kripke et al. 2006; Benedetti, Dallaspezia et al. 2007; Kripke, Nievergelt et al. 2009; Mansour, Talkowski et al. 2009; Lamont, Coutu et al. 2010; Sjoholm, Backlund et al. 2010; Soria, Martinez-Amoros et al. 2010). In addition, rhythm disruptions and sleep disturbances, are common in BPD and often precipitate manic or depressive episodes (Lamont, Legault-Coutu et al. 2007; Harvey 2011) . Interestingly, effective treatments for BPD also affect circadian rhythms. Interpersonal and Social Rhythm Therapy (IPSRT) relies on the stabilization of social

rhythms following an episode, including sleep/wake cycle, meal times, and social interactions (Frank, Swartz et al. 2000).

Mouse models have further strengthened the link between circadian rhythms and BPD. Mice bearing a point mutation in the *Clock* gene were generated by an ENU mutagenesis screen to identify molecules involved in circadian rhythms (Vitaterna, King et al. 1994). This point mutation causes a deletion of exon 19 in the CLOCK protein (CLOCKΔ19), which contains the transactivation domain, resulting in a dominant negative CLOCK function. CLOCKΔ19 can heterodimerize with BMAL1 and bind to E-box elements, but can no longer activate transcription. *Clock*Δ19 mice have a complete behavioral profile which is very similar to human mania (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007). These include hyperactivity, decreased anxiety-related and depression-related behavior, and increased preference for rewarding stimuli (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007).

Previous studies from our lab have identified an important role for the ventral tegmental area (VTA) in the development of the *Clock*Δ19 phenotype. The VTA provides dopaminergic input to the nucleus accumbens (NAc), making up the mesolimbic dopamine circuit. This circuit is known to play important roles in natural and drug-induced reward, as well as mood regulation (Self and Nestler 1995; Nestler and Carlezon 2006). When CLOCK levels are decreased in the VTA of wild type (WT) animals using a specific shRNA-containing adeno-associated virus (AAV) , behaviors similar to *Clock*Δ19 mice, including hyperactivity and decreased anxiety, develop (Mukherjee, Coque et al. 2010). Conversely, when viral mediated gene transfer is utilized to express a functional CLOCK in the VTA of *Clock*Δ19 mice, locomotor activity and anxiety-

related behavior are restored to WT levels (Roybal, Theobold et al. 2007). Interestingly, the *Clock*Δ19 mice also have increased firing and bursting of VTA dopamine neurons. Moreover, knockdown of CLOCK in the VTA using an AAV-shRNA caused an increase in cell firing, specifically in infected dopamine cells, suggesting that the *Clock*Δ19 mutation has a cell-intrinsic effect on cell firing (Mukherjee, Coque et al. 2010). This increase in VTA dopamine neuron firing rate also plays a role in the development of the *Clock*Δ19 manic-like behaviors, as decreasing the firing rate of *Clock*Δ19 VTA dopamine neurons reverses several of their abnormal behaviors, including locomotor and anxiety-related behavior (Coque, Mukherjee et al. 2011).

Chronic administration of the mood stabilizer lithium has therapeutic effects on the mood-related behavior of *Clock*Δ19 mice, restoring anxiety and depression-related behaviors to WT levels (Roybal, Theobold et al. 2007); therefore, *Clock*Δ19 mice also have predictive validity as a model of mania. Interestingly, this behavioral rescue is selective to *Clock*Δ19 mice, as lithium treatment either has no effect or an opposite effect on WT animal behavior. Despite its use a therapeutic agent for over 50 years, lithium's mechanism of action still remains unclear. The *Clock*Δ19 mice provide a unique opportunity to study lithium's mechanism of action, because lithium treatment does not have the same effects on healthy subjects as BPD patients, similar to what is observed in the *Clock*Δ19 mice. Most studies on lithium's mechanism of action have been performed in WT animals, which may be difficult to interpret because these mice model a "normal" human control, which does not respond to lithium treatment in the same manner as a BPD patient. In the *Clock*Δ19 mouse, lithium may ameliorate behavior in part by affecting the VTA because lithium administration also selectively decreases VTA

dopamine cell firing in the *Clock*Δ19 mutants, which has shown to be sufficient to partially reverse their behavior (Coque, Mukherjee et al. 2011).

Because CLOCK is a transcription factor, it is likely that altered transcription of target genes in the VTA plays a role in the development of their manic-like behaviors. Microarray analysis of VTA tissue from *Clock*Δ19 mice and WT littermates revealed altered transcription of many genes involved in dopaminergic transmission (McClung, Sidiropoulou et al. 2005). One of these genes was the neuropeptide transmitter, *cholecystokinin* (CCK), which was decreased in the VTA of *Clock*Δ19 mice (McClung, Sidiropoulou et al. 2005). CCK is a member of the gut-brain family of neuropeptides. The sulphated carboxy terminal octapeptide, CCK-8S, is the most commonly expressed form in the brain, with larger forms expressed in the gut. There are two major sub-types of CCK receptors, CCK_A and CCK_B. CCK_B is the more common subtype, found throughout the cortex, hippocampus, NAc, caudate putamen, and olfactory regions (Carlberg, Gundlach et al. 1992; Honda, Wada et al. 1993). CCK_A is found in smaller numbers in some of these regions, as well as the hypothalamus (Carlberg, Gundlach et al. 1992; Honda, Wada et al. 1993). The primary action of CCK in the brain is thought to be mediated through the CCK_B receptor, which has been shown in cultured striatal neurons to increase intracellular calcium, which may be through activation of phospholipase-C (Miyoshi, Kito et al. 1991).

Within the VTA and the substantia nigra, CCK has been found to be highly co-localized with dopamine, specifically within dopaminergic neurons that project to the NAc with 40-80% of the cells co-expressing dopamine and CCK (Hokfelt, Rehfeld et al. 1980; Lanca, De Cabo et al. 1998). At VTA dopaminergic terminals, CCK is co-released

with dopamine, specifically upon burst firing (Ghijsen, Leenders et al. 2001). CCK acts as a negative modulator of dopaminergic transmission *in vivo*, as infusions of CCK-8S into the NAc inhibit K+-stimulated dopamine release and reduce extracellular dopamine concentrations (Voigt and Wang 1984). This effect is mediated by CCK_B receptors as K+-stimulated dopamine release can be attenuated by the intracerebroventricular (i.c.v.) administration of CCK_B specific compounds t-butoxycarbonyl tetrapeptide and unsulfated CCK-8, while the effects of CCK-8S on dopamine release cannot be prevented by the administration of the CCK_A antagonist L-364178 (Altar 1989). CCK_B receptors are thought to inhibit D2 dopamine receptor function on GABAergic medium spiny neurons (MSNs) of the NAc, though whether or not this is the sole action of CCK_B receptors unclear (Tanganelli, Fuxe et al. 2001). These neurons project back to the VTA, providing negative regulation of dopaminergic activity. D2 receptors inhibit MSN activity; thus, CCK_B receptor activation would increase GABAergic inhibition of VTA dopamine neurons. It is therefore possible that the increased VTA dopamine activity observed in the *Clock*Δ19 mice is due in part to the reduced levels of CCK in the VTA.

In addition to its effects on dopaminergic transmission, CCK also modulates many behaviors that are disrupted in the *Clock*Δ19 mice. Microinjections of CCK-8S into the anterior nucleus accumbens inhibit dopamine-induced hyperlocomotion (Crawley 1992). This effect of CCK-8S is blocked using the selective CCK_B receptor antagonist, CI-988 (Crawley 1992). Systemic administration of caerulein, a general CCK receptor agonist, in mice resulted in anxiety-like behavior in the elevated plus maze and open-field tests (Harro, Pold et al. 1990). These effects have been replicated in both mice and rats using CCK or specific CCK_B receptor agonists in many behavioral measures including

the elevated plus maze, open-field test, and acoustic startle (Rex, Barth et al. 1994; Fendt, Koch et al. 1995; van Megen, Westenberg et al. 1996). CCK-induced anxiety in model organisms can be abolished by pre-treatment with the CCK_B receptor antagonist L-365260, further suggesting that CCK is anxiogenic via its action at CCK_B receptors (Mannisto, Lang et al. 1994; van Megen, Westenberg et al. 1996). Conversely, subcutaneous administration of selective CCK_B receptor antagonists PD134308 and PD135158 has anxiolytic effects in the dark/light test in mice (Hughes, Boden et al. 1990). In fact, intravenous administration of CCK_4, a CCK_B agonist, produces panic-like symptoms in healthy human volunteers (de Montigny 1989). Panic disorder patients are also more sensitive to CCK_4 administration, and described its effects as identical to that of their naturally occurring panic attacks (Abelson and Nesse 1994; van Megen, Westenberg et al. 1996).

The potential role of CCK and CCK_B receptors in depression-related behavior has not been as well characterized as anxiety; however, CCK may have a "pro-depressant" effect. Rats subjected to social defeat were shown to have an increase in cortical CCK that was correlated with increased immobile time in the forced swim test (Becker, Zeau et al. 2008). Treatment with the CCK_B receptor antagonist CI-988 normalizes immobile time in defeated rats, similar to what was observed with administration of the antidepressant imipramine (Becker, Zeau et al. 2008). The CCK_B receptor antagonist L-365,260 has also been shown to have antidepressant effects in mice subjected to the forced swim test (Hernando, Fuentes et al. 1994). CCK also appears to be involved in reward and motivational behavior, as intra-accumbens administration of CCK_B receptor agonists decreases the break point on a progressive ratio schedule for

amphetamine self administration, indicative of a decreased reward magnitude for amphetamine (Rotzinger, Bush et al. 2002).

In summary, CCK appears to decrease locomotor activity, be positively correlated with anxiety and depression-related behavior, and decreases reward magnitude. It remains unclear which brain regions are involved in these effects of CCK, though the mesolimbic dopamine circuit is known to contribute to mood and reward related behaviors. We have previously observed a decrease in *Cck* mRNA levels in the VTA of *Clock*Δ*19* mice, and the behavioral phenotype of *Clock*Δ19 mice correlates well with a decrease in CCK levels and function. It is unclear whether this observed decrease in *Cck* mRNA is a direct result of the *Clock*Δ19 mutation; however, the *Cck* promoter contains a conserved E-box element that may be a binding site for the CLOCK protein (Hansen 2001). We therefore hypothesize that the *Cck* gene is a CLOCK target, and that decreased CCK levels in the VTA play a role in the development of *Clock*Δ19 manic-like behaviors. To test this hypothesis we utilized chromatin immunoprecipitation to assess CLOCK binding at the *Cck* promoter, and an AAV-shRNA directed against *Cck* to decrease CCK levels specifically in the VTA of WT animals and examine its effects on mood-related behavior. Because lithium treatment rescues *Clock*Δ19 behaviors, and this rescue resembles a restoration in CCK function, we also examined whether lithium affected *Cck* expression in the VTA of *Clock*Δ19 mice, and investigated whether or not *Cck* is important for lithium's therapeutic action in *Clock*Δ19 mice.

Materials and Methods:

Animals and Housing *Clock*Δ19 mutant mice were created by *N*-ethyl-*N*-nitrosurea mutagenesis and produce a dominant-negative CLOCK protein defective in transcriptional activation activity as described (King, Vitaterna et al. 1997). For all experiments using *Clock*Δ19 mutants, 8 to 16 week old adult male mutant (*Clock*Δ19*;* Mut*)* and wild-type (WT) littermate controls on a mixed BALBc; C57BL/6 background were used. Mice were group housed in sets of 2-4 per cage on a 12:12 h light/dark cycle (lights on 6:00 a.m., lights off at 6:00 p.m) with food and water provided *ad libitum*. To examine the effects of *Cck* knock-down on wild-type behavior, 8 to 10-week old adult male C57BL/6 mice from Jackson laboratories were used. All mouse experiments were performed in compliance with National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committees of UT Southwestern Medical Center.

Lithium Administration Lithium treated mice received 600 mg/l of LiCl in drinking water for 10 days prior to behavioral testing, and throughout the course of the testing. This administration results in a stable serum concentration of lithium in the low therapeutic range for human patients (0.41±0.06 mmol/l), with little to no adverse health consequence (Roybal et al, 2007).

Chromatin Immunoprecipitation (ChIP) ChIP assays were performed according to methods described previously (Tsankova, Kumar et al. 2004; Enwright, Wald et al. 2010). Briefly, 1mm VTA containing midbrain sections taken from *Clock*Δ19 mice or wild type (WT) littermates were cross-linked in 1% formaldehyde for 15 minutes. Cross-linking was then quenched by the addition of glycine at a final concentration of 0.125M

for 5 minutes. Chromatin was sheared to 0.2 to 1kb fragments by sonication and cleared with Protein A beads (Thermo Scientific #22811). For each pull-down, approximately 60-100μg of chromatin was used. Chromatin was incubated, rotating overnight at 4°C, 5-10μg of one of the following antibodies: Clock (Santa Cruz Biotechology #H-276), acetylated histone H3 (Millipore #06-599), IgG (Millipore #12-370) , trimethyl-histone H3 Lysine 4 (Millipore #07-473), and MLL1 (Bethyl Labs #A300-086A). The antibody-chromatin complexes were immunoprecipitated using Protein A beads plus according to manufacturer's instructions. Following reverse cross-linking of input and immunoprecipitated samples, quantitative PCR was performed to determine levels of protein binding at promoters of interest using primers against the proximal *Cck* promoter. Input DNA and immunoprecipitated DNA were amplified in duplicate in the presence of SYBR Green on the ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Relative quantification of template DNA was performed using the fold enrichment method.

Reporter and Expression Plasmids: The Wild-Type Cck luciferase reporter was generated previously (Enwright, Wald et al. 2010). The wild-type (WT) *Cck* promoter-luciferase reporter was prepared by inserting an approximately 200-bp PCR fragment into the pGL3-luc vector (Promega). This fragment was obtained from mouse genomic DNA (primers: 5′-TATCCTCATTCACTGGGACGC-3′ upstream, and 5′-TACCTTTGGATGGGGAAATCG-3′ downstream) and initially inserted into pGEM-T Easy vector (no. A1360; Promega). The promoter fragment was then cloned into the *Kpn*1/*Xho*1 restriction enzyme sites of pGL3-luc. Mutagenic primers were designed to

induce point mutations in the putative E-box site in the *Cck* promoter (sense primer: 5 '-GTGGCTGCCTCTGAGCCCGGGTCCTGCTGGACTGCG-3', antisense primer: 5'-CGCAGTCCAGCAGGACCCGGGCTCAGAGGCAGCCAC-3') using the Stratagene QuikChange II Site-Directed Mutagenesis Kit (cat. # 200555). This mutation changed the sequence of the E-box from CACGTG to CCCGGG, which resulted in the insertion of a diagnostic *Xma1* site. The CLOCK and BMAL1 expression constructs contain full length sequences inserted into the multiple cloning site of pCDNA 3.1.

Luciferase Assays: Cell culture and performance of the luciferase assay were carried out as described previously (Enwright, Wald et al. 2010). Briefly, rat pheochromocytoma (PC12) cells were cultured in Dulbeco's modified Eagle's Medium F-12 supplemented with 10% horse serum, 5% fetal bovine serum, and 1% penicillin/streptomycin in a 37°C, 5% CO_2 incubator. PC12 cells were transfected by electoporation (BTX 360) with 5 µg of the expression constructs. Three days post-transfection, cells were lysed with lysis buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1% Triton X-100, pH 7.8, 1 mM DTT) and centrifuged to clear cellular debris. 30 µl of the resulting lysate was combined with 140 µl of luciferase assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 1 mM ATP, 1 mM potassium phosphate, 1 mM coenzyme A, pH 7.8). A FLx-800 microplate fluorescence reader was used to measure luminescence after an automated injection of 40 µL of 1 mM luciferin per well. Luciferase activity was normalized to total protein levels as measured by Bio-Rad protein assay.

Construction of Cck ShRNA and Virus Purification: A small hairpin RNA (shRNA) directed against *Cck* was designed using previously published criteria (Mukherjee, Coque

et al. 2010). For the Cck shRNA, a 15 base pair sequence in the coding region of the *Cck* gene (5'-CTTGAGCGGTTCGG-3') was identified as a target region. A previously published scrambled RNA sequence (5'-CGGAATTTAGTTACGGGGATCCAC-3') that has no known sequence similarities was used as a negative control. An antisense sequence of selected region and a miR23 loop of 10 nucleotides (CTTCCTGTCA) was added to the 5' end of these sequences. The annealed oligonucleotides were cloned into an adeno-associated virus (AAV) plasmid expressing enhanced green fluorescent protein (Stratagene, La Jolla, CA). Viral production was carried out using a helper-free triple transfection method. In brief, HEK-293 cells were cultured in seven 150 X 25 mm cell culture dishes and transfected with pAAV-Scr-shRNA or pAAV-Cck-shRNA, pHelper, and pAAV-RC plasmids using a standard calcium phosphate method. Cells were collected, pelleted by centrifugation, and resuspended in a freezing buffer (0.15M NaCl, 50mM Tris, pH 8.0) approximately 72 hours after transfections. The cells were lysed by 5 freeze thaw cycles. Benzonase was then added at a final concentration of 50 U/ml to the lysate and incubated at 37°C for 30 minutes. Afterwards, the lysate was added to a centrifuge tube containing an iodixanol step gradient (15%, 25%, 40%, and 60%). The gradient was spun at 60,000 g for 90 minutes at 4°C. The 40% fraction was then added to a heparin affinity column and then washed with 0.1 M NaCl and eluted with 0.4 NaCl in PBS using Amicon Ultra-15Centrifugal Filter Units (Millipore). The final purified virus was stored at -80 °C. Additional viral production was also carried out by Dr. R. Jude Samulski and the UNC Gene Therapy Vector Core.

Laser Capture Microdissection: Laser capture microdissection (LCM) to assess levels of *in vivo Cck* knockdown were performed as in previous studies (Mukherjee et al.,

2010). Briefly, brains from mice infected with AAV scrambled or Cck shRNA, were frozen and sliced into 7μm sections. The sections were placed onto slides (Arcturus, Sunnyvale, California) and stored at -80°C until further processing on the Arcturus LCM instrument. The LCM slides were dehydrated in a series of 70%, 90%, 95%, and 100% ethanol for 30 seconds, followed by 30 seconds of xylene. The slides were then air-dried and mounted in the LCM. Fluoresecence microscopy was used to visualize infected regions because of the viral expression of GFP. At least 3,000 GFP-positive were captured from each mouse. Following sample, collection, the PicoPure RNA extraction kit (Molecular Devices, Sunnyvale, California) was used to purify RNA per manufacturer's instructions.

Stereotaxic Surgery: Mice were anesthetized with a mixture of ketamine (62.5 mg/kg body weight) and xylazine (10 mg/kg body weight) in saline (0.9% NaCl). Bilateral stereotaxic injections into the VTA (from bregma: angle 7°, AP -3.2mm, Lat +1.0, DV -4.6) of 1μl AAV-Cck-shRNA or AAV-Scr (1x 10^{12} infectious particles/ml) were performed using a 33 gauge Hamilton syringe (Hamilton, Reno, NV). The speed of injection was 0.1 μl/minute, after which the needle was kept in place for 5 minutes before it was withdrawn. Mice recovered for three weeks in their home cage before behavioral testing or treatment.

Immunohistochemistry and validations of injections and infections Mice were anesthetized with a mixture of Nembutal (50 mg/kg) in saline, followed by transcardial perfusion with 4% paraformaldehyde in 1x PBS (1 mM KH2PO4, 10 M Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and placed in 30% glycerol in 1X PBS. Brains were

sliced into 30μm sections using a microtome (Leica, Wetzlar, Germany) and immunohistochemical staining was carried out using standard procedures with the following antibodies: tyrosine hydroxylase (TH) mouse monoclonal antibody (1:5000; T2928; Sigma, St. Louis, MO), and GFP rabbit polyclonal antibody (1:20,000; ab290; AbCam, Cambridge, MA). Secondary antibodies (1;400 anti-mouse or rabbit conjugated with Alexa 488/rhodamine) were purchased from Invitrogen (Carlsbad, CA). Immunostained sections were mounted using Vectashield (Vector Labs, Burlingame, CA) and observed with an epifluorescence microscope to identify the location of viral injection and levels of infection. Sections were visualized using 4, 10, and 20x objectives. VTA cells that displayed more than 40% of EGFP co-localization with TH antibody staining were included for behavioral analysis. Injections that were visibly outside the VTA were excluded from the study.

Behavioral Assays

Locomotor Response to Novelty: Mice were placed into individual automated locomotor activity chambers that were equipped with infrared photobeams (San Diego Instruments, San Diego, CA). Activity measurements commenced upon the first beam break and were measured continuously and data was collected in five minute blocks over a period of two hours.

Elevated Plus Maze: The plus maze apparatus consisted of closed and open arms (all arms are 30 x 5 cm, with 25 cm tall walls on the closed arms). Mice were placed in the center of an elevated plus maze and the time spend in the open arms, closed arms, and center of the maze, along with the number of entries into the open and closed arms of the maze were determined by Ethovision 3.0 video tracking software (Noldus, Leesbrg, VA).

Time spend on the open arm and percent of entries in to the open arm were used to determine anxiety-related behavior. The apparatus was cleaned and allowed to dry between every mouse.

Dark/Light Test: The dark/light apparatus is a 2 chambered box (25 cm x 26 cm for each side, Med Associates, St. Albans, VT), one side of which was kept dark, and the other side brightly lit by a fluorescent bulb at the top of the chamber. Mice were allowed to habituate to the dark side of the box for two minutes. Following the habituation period, the door between the compartments was opened and they were allowed to freely explore both sides of the apparatus for 10 minutes. Anxiety-like behavior was measured as the percent of time spent in the light side.

Forced Swim Test: Mice were placed in 4 liter Pyrex glass beakers were filled with 3 liters of water at 21-25°C for 6 minutes. All test sessions were recorded by a video camera from the side of the beakers. Water was changed between subjects. The video was analyzed and scored by an observer blind to the genotypes and treatment groups. After a 2 minute habituation time, latency to immobility was determined as the first cessation of movement. Total immobility was measured during the last four minutes of the test and was measured as time spent without movement except for a single limb paddling to maintain flotation.

Quantitative PCR: cDNA or purified genomic DNA was mixed with buffers, primers, SYBR green, and hot start Taq polymerase in a master mix prepared by a manufacturer (Applied Biosystems, Foster City, CA). Using a Real-Time PCR machine (7500 Real Time PCR machine, Applied Biosystems) PCR reactions were run followed by a

dissociation reaction to determine specificity of the amplified product. The amount of gene expression was quantified using the ΔΔCt method as previously described.

Primer Sequences: *Gapdh*: Forward – 5'-AACGACCCCTTCATTGAC-3', Reverse- 5'-TCCACGACATACTCAGCAC-3'

Cck:: Forward- 5'-ACTGCTAGCGCGATACATCC-3' , Reverse- 5'-TTCGTAGTCCTCGGCACTGC-3'

Cck Promoter: Forward- 5'-CTTGGGCTAGCCTCATTCACTG-3', Reverse-5'-TTAAATAGCTCCTCCCGGTTCG-3'

Statistical analysis: For the comparison of two groups, two-tailed unpaired Student's t tests were used. When more than one factor was examined simultaneously, two-way ANOVAs were performed followed by Bonferroni post-tests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

CCK is a CLOCK target gene in the VTA

To determine if the previously observed decrease in *Cck* mRNA levels was a direct or indirect result of the *Clock*Δ19 mutation, we examined whether or not CLOCK bound to the *Cck* promoter. The *Cck* promoter contains an E-box element (CANNTG) that is conserved in mouse, rat and human, and is part of a 110 bp proximal promoter region that is over 80% conserved from mouse to human (Hansen 2001). In addition to the E-box, there is also a GC-rich region, CRE/TRE site, and putative TATA box (Figure 2-1a; (Hansen 2001). To assess potential CLOCK binding at the *Cck* promoter chromatin immunoprecipitation (ChIP) assays, were carried out on VTA-containing midbrain tissue from WT and *Clock*Δ19 littermates. CLOCK was found to be enriched at the *Cck* promoter above background in both genotypes (~4-fold in WT, ~6-fold in *Clock*Δ19) with no significant difference between the two (Figure 2-1b). To further elucidate the role of CLOCK in the transcriptional regulation of the *Cck* gene, luciferase assays were performed in PC12 cells using *Cck*-luc reporter plasmids containing approximately 300 bp of the *Cck* proximal promoter region with either an intact or a mutated E-box site. When this reporter plasmid was co-transfected into cells along with CLOCK and BMAL1 expression constructs, a significant increase in *Cck*-luc reporter activity was observed when the E-box was intact (Figure 2-1c). When the E-box was mutated, the ability of transfected CLOCK/BMAL1 to induce *Cck*-luc reporter activity was abolished (Figure 2-1c). These results suggest that CLOCK acts as a positive regulator of the *Cck* gene in an E-box dependent manner and that the decrease in *Cck* gene expression observed in the *Clock*Δ19 mice is a direct result of the *Clock*Δ*19* mutation.

Knockdown of CCK in the VTA of WT animals results in a manic-like phenotype

To determine if decreased CCK levels play a role in the development of the manic-like behaviors of the *Clock*Δ19 mice, an AAV-shRNA directed specifically against *Cck* was generated (AAV-Cck-shRNA). Stereotaxic injection of AAV-Cck-shRNA into the VTA of C57/BL6 mice was found to cause a significant (~6) decrease in *Cck* mRNA levels (Figure 2-2b). Once the ability of AAV-Cck-shRNA to knock-down *Cck* levels *in vivo* was determined, the effects of reduced VTA *Cck* levels on behavior was examined. Three weeks after stereotaxic injection of AAV-Cck-shRNA or a scrambled shRNA control (AAV-Scr) into the VTA of C57/BL6 mice, they were subjected to a battery of behavioral tests. We first determined the effect of *Cck* knock-down on locomotor activity. When exposed to a novel environment, mice injected with AAV-Cck-shRNA were significantly hyperactive compared to those injected with AAV-Scr over the course of two hours (Figure 2-3a). To determine the effects of decreased *Cck* in the VTA on anxiety related behavior, the mice were then subjected to two behavioral tests: the elevated plus maze and dark/light box. Mice injected with AAV-Cck-shRNA were found to have decreased anxiety-related behavior when compared to AAV-Scr controls. AAV-Cck-shRNA mice spent significantly more time in the open arms of the elevated plus maze and in the light side of the dark/light box (Figure 2-3b,c). These results correspond well to previous studies with systemic administration of CCK_B receptor antagonists, which were found to be anxiolytic (Hughes, Boden et al. 1990). We then determined the effects of *Cck* knock-down on depression-related behavior by subjecting animals to the Porsolt forced swim test. Decreased immobile time was observed in mice injected with AAV-Cck-shRNA compared to AAV-Scr control mice (Figure 2-3d). An increase in

latency to first bout of immobility was also observed in AAV-Cck-shRNA animals as well (Figure 2-3e), indicating that a knock-down of *Cck* in the VTA also decreases depression-related behavior, which could be interpreted as an "elevated mood state." Taken together, the behavior of AAV-Cck-shRNA mice is very similar to the *Clock*Δ19 animals.

Lithium selectively restores *Cck* expression levels in *Clock*Δ19 mice

Because we were able to determine that a decrease in *Cck* is sufficient to induce manic-like behaviors (Figure 2-3), and lithium treatment rescues *Clock*Δ19 mutant behavior, we investigated whether or not *Cck* was a potential lithium target gene. RNA was isolated from the VTA of *Clock*Δ19 and WT mice receiving either chronic lithium (600 g/L in drinking water) or normal drinking water and qPCR was performed to assess *Cck* mRNA levels. As reported previously, *Cck* levels were decreased in control *Clock*Δ19 mice relative to WT littermates (Figure 2-4, (McClung, Sidiropoulou et al. 2005). When *Clock*Δ19 mice were administered lithium, *Cck* mRNA levels were restored to near WT (Figure 2-4). Interestingly, lithium treatment had no detectable effect on WT *Cck* mRNA levels (Figure 2-4). This mimics what is observed at the behavioral levels, as lithium treatment does not have the same effects on mood-related behavior in WT animals as in *Clock*Δ19 mice (Roybal, Theobold et al. 2007).

Increased *Cck* levels in the VTA are required for the therapeutic effects of lithium in *Clock*Δ19 mice

Lithium has been previously found to rescues anxiety and depression-related behavior in *Clock*Δ19 mice, which resembles what could be expected with increased CCK function. When it was determined that lithium selectively restored *Cck* mRNA to

WT levels in the VTA *Clock*Δ19 mice, we hypothesized that *Cck* may be an important target for lithium's therapeutic action in the *Clock*Δ19 mice. To test this hypothesis, we injected AAV-Cck-shRNA or AAV-Scr into the VTA of *Clock*Δ19 mice. These mice then received chronic lithium treatment or normal drinking water to determine if lithium could still rescue *Clock*Δ19 anxiety and depression related behavior when *Cck* levels were unable to be increased. Similar to WT animals, *Clock*Δ19 mice injected with AAV-Cck-shRNA were hyperactive compared to AAV-Scr controls (Figure 2-5a). As has been reported previously, lithium treatment had no detectable effect on locomotor activity in animals injected with either virus; therefore, effects on the other behavioral tests were due exclusively to changes in anxiety and depression-realted behavior (Figure 2-5a; (Roybal, Theobold et al. 2007). To assess whether an increase in *Cck* was important for lithium's restoration of *Clock*Δ19 anxiety-related behavior, mice were subjected to the elevated plus maze and dark/light box. Interestingly, untreated mice infected with AAV-Cck-shRNA showed no detectable difference in baseline anxiety behavior compared to AAV-Scr in either measure of anxiety-related behavior. This also indicates that changes in locomotor activity observed in AAV-Cck-shRNA animals did not confound measures of mood-related behavior. As has been observed previously, lithium decreased exploratory behavior, as indicated by a decrease in open arm time on the elevated plus maze and decreased time spent in the light side of the dark/light box, in AAV-Scr injected *Clock*Δ19 mice (Figure 2-5b,c; (Roybal, Theobold et al. 2007). However, when *Cck* was knocked-down in the VTA, lithium was no longer able to rescue anxiety-related behavior in the *Clock*Δ19 mice, as AAV-Cck-shRNA injected animals receiving normal drinking water or lithium displayed no detectable differences in time spent in the open

arms of the elevated plus maze or light side of the dark/light box (Figure 2-5b,c). Following assessment of whether lithium required increased *Cck* levels to exert its therapeutic effects on anxiety-related behavior, the mice were then subjected to the forced swim test. Similar to what was observed in the anxiety-related behavioral tests, untreated mice infected with AAV-Cck-shRNA showed no detectable difference in baseline depression-related behavior when compared to AAV-Scr as shown by total immobile time and latency to immobility (Figure 2-5d,e). Lithium treatment , resulting in an increase in total immobile time and a decreased latency to immobility in the forced swim test in AAV-Scr infected *Clock*Δ19 mice, similar to what has been previously determined (Figure 2-5d,e(Roybal, Theobold et al. 2007)). Lithium again failed to have the same "anti-manic" effect when administered to AAV-Cck-shRNA infected *Clock*Δ19 mice, which had no detectable difference in total immobility or latency to immobility from untreated mice which were infected with AAV-Cck-shRNA (Figure 2-5d,e). These results suggest that an increase in *Cck* levels is required in order for lithium to have therapeutic effects in *Clock*Δ19 mice.

Regulation of Histone H3K4me3 at the Cck promoter in *Clock*Δ*19* mice by the CLOCKΔ19 protein and lithium treatment

After determining the importance of *Cck* in the development and treatment of manic like behaviors, we further examined the mechanism of the decreased *Cck* levels observed in *Clock*Δ19 mice and investigated how lithium treatment may regulate the *Cck* gene. Previous studies have found that CLOCK activates transcription via interactions with the histone methyltransferase mixed lineage leukemia 1 (MLL1), which then causes tri-methylation of histone H3 at lysine 4 (H3K4me3), which then allows for transcriptional

activation of CLOCK target genes (Katada and Sassone-Corsi 2010). In the same study, it was also found that CLOCKΔ19 fails to associate with MLL1, and this failure is responsible for its dominant negative function (Katada and Sassone-Corsi 2010). These studies were performed *in vitro*, so we set out to examine if the decrease in *Cck* in *Clock*Δ19 mice was caused by this mechanism, and whether lithium treatment had any effect on this chromatin modification as well. Chromatin immunoprecipitation assays were carried out using an antibody against H3K4me3 in *Clock*Δ19 mice and WT controls receiving either water or lithium (Figure 2-6a). There was a significant decrease in H3K4me3 at the *Cck* promoter in *Clock*Δ19 mice compared to WT animals. Lithium treatment caused a restoration of H3K4me3 levels to those near WT, while treatment had no detectable effect on WT animals (Figure 2-6a). This is similar to what was observed at the *Cck* mRNA level (Figure 2-4). The decrease in H3K4me3 in *Clock*Δ19 animals corresponds with the predicted inability of CLOCKΔ19 to interact with MLL1. We performed ChIP assays to determine if there were decreases in MLL1 levels at the *Cck* promoter in *Clock*Δ19 mice and if lithium had any affect on MLL1 association with the *Cck* promoter which could cause the increase in H3K4me3 levels observed in treated *Clock*Δ19 mice. We found a decrease in MLL1 levels at the *Cck* promoter in *Clock*Δ19 mice when compared to WT animals (Figure 2-6b). Following lithium treatment, there were increased levels in MLL1 at the *Cck* promoter in *Clock*Δ19 mice, though this did not reach significance (Figure 2-6c). This suggests that MLL1 may contribute to the increase in H3K4me3 levels at the *Cck* promoter in lithium treated *Clock*Δ19 mice, though other histone methyltranferases may be involved. Lithium treatment had no significant effect on MLL1 levels at the Cck promoter in WT animals (Figure 2-7a). To

determine how lithium treatment resulted in recruitment of MLL1 and other potential methyltransferases to the *Cck* promoter, we assessed, using ChIP assays, whether there were any changes in CLOCK binding to the *Cck* promoter in *Clock*Δ19 mice following administration of lithium. A significant reduction in CLOCK at the *Cck* promoter was observed in *Clock*Δ19 mice receiving lithium treatment, suggesting that lithium causes another DNA-binding protein to compete with CLOCK at the *Cck* promoter, either for the E-box element or a nearby binding element such as the CRE site (Figure 2-6d). Interestingly, lithium treatment caused a modest but insignificant decrease in CLOCK levels at the *Cck* promoter in WT animals, suggesting that this molecular effect of lithium treatment may be somewhat non-specific to genotype (Figure 2-7b).

Discussion

Our results identify the peptide neurotransmitter *Cck* as a novel target of the CLOCK protein in the VTA. CLOCK acts as a positive regulator of the *Cck* gene via its interactions at the E-box element of the *Cck* promoter (Figure 2-1). This finding indicates that the decrease in *Cck* mRNA observed in the VTA of *Clock*Δ*19* mice is a direct result of the *Clock*Δ19 mutation. We have also identified *Cck* to be a CLOCK target in the VTA that is involved in the development of the manic-like phenotype observed in the *Clock*Δ*19* mice. AAV-mediated knockdown of *Cck* specifically in the VTA results in hyperactivity, decreased anxiety-related behavior, and a decrease in depression-related behavior (Figure 2-3). These changes in behavior are similar to the *Clock*Δ*19* mice, indicating that the *Clock*Δ*19* phenotype is due in part to a decrease in *Cck* levels. Interestingly, the behavioral effects of *Cck* knockdown differ somewhat from those observed in mice that have AAV-mediated knockdown of CLOCK specifically in

the VTA (Mukherjee, Coque et al. 2010), which results in hyperactivity and decreased anxiety, but an increase in depression related behavior when compared to control animals. Possibilities for this discrepancy include that knocking down *Cck* in the VTA directly mimics one of the molecular effects of the presence of the dominant negative CLOCKΔ19, as opposed to a decrease in CLOCK levels cause by an AAV-Cck-shRNA. Additionally, there was modest decrease in *Cck* levels in the AAV-Clock-shRNA infected cells (unpublished observations), whereas in AAV-Cck-shRNA infected cells there was a more robust decrease (Figure 2-2), which may have been sufficient to elicit changes in depression related behavior.

We also identified that *Cck* is involved in lithium's therapeutic effects in *Clock*Δ*19* mice, as chronic lithium administration resulted in a restoration of *Cck* mRNA to WT levels in the VTA without having any detectable effect on WT *Cck* levels (Figure 2-4). This finding makes *Cck* a particularly promising target as lithium treatment selectively rescues *Clock*Δ*19* mouse behavior while having little effect on WT animals (Roybal, Theobold et al. 2007). This is similar to what is observed in the human population, as lithium is effective in the treatment of bipolar mania, but has little or variable effect on a normal patient population (Grandjean and Aubry 2009; Malhi, Adams et al. 2009). Therefore, the selective regulation of *Cck* by lithium may be of particular therapeutic relevance. We found that this increase in *Cck* is necessary for lithium's "anti-manic" effects in the *Clock*Δ*19* mice, as *Clock*Δ*19* mice injected with AAV-Cck-shRNA into the VTA no longer respond to lithium treatment in the same manner as AAV-Scr controls (Figure 2-5).

Investigations into the mechanism of how lithium is regulating the *Cck* gene found that it regulates levels of H3K4me3, a histone modification highly associated with transcriptional activation, in the *Clock*Δ*19* mice. In *Clock*Δ*19* mice there is a decrease in H3K4me3 levels when compared to WT mice (Figure 2-6a). This is due in part to the inability of the mutant form of CLOCK to associate with MLL1, which was also found to be decreased at the *Cck* promoter in *Clock*Δ*19* mice relative to WT littermates (Figure 2-6b). Though this lack of association was thought to underlie the dominant negative function of CLOCKΔ19 based on *in vitro* findings, we provide the first *in vivo* evidence that this is indeed the case. Lithium treatment causes a restoration of H3K4me3 levels at the *Cck* promoter in *Clock*Δ*19* mice while not affecting WT animals (Figure 2-6a). This rescue of H3K4me3 is accompanied by a nonsignificant increase in MLL1 at the *Cck* promoter (Figure 2-6c), which indicates that it may contribute to lithium's effects on H3K4me3 in the *Clock*Δ*19* mice. It is likely that other histone methyltransferases known to regulate H3K4me3, such as other MLL family members or Set1a/b, are also involved in the increase of this histone modification following lithium treatment (Ge 2012). Lithium treatment also causes a decrease in CLOCK levels at the *Cck* promoter in the *Clock*Δ*19* mice, suggesting that another protein or transcriptional-activation complex is competing for with CLOCKΔ19 for *Cck* promoter occupancy (Figure 2-6d). This could be at the E-box element, or at another nearby site, such as the CRE site. Interestingly, lithium treatment also causes slight, but non-significant, decrease in CLOCK levels at the *Cck* promoter in WT animals, suggesting that a similar mechanism to the *Clock*Δ*19* mice may be occurring (Figure 2-7b). This competition results in the removal of the dominant

negative CLOCKΔ19 in mutant mice, but not in WT animals, which may explain why *Cck* levels are selectively restored in *Clock*Δ*19* mice.

In conclusion, we have identified a novel CLOCK target gene, *Cck*, and that the inability of the CLOCKΔ19 protein to regulate the *Cck* gene is important for the development of manic-like behaviors in the *Clock*Δ*19* mice. This decrease in *Cck* transcription is due in part to the inability of CLOCKΔ19 to interact with the histone methyltransferase MLL1, which results in a decrease of H3K4me3. Additionally, we have determined that an increase in *Cck* mRNA levels is necessary for lithium's therapeutic action in the *Clock*Δ*19*. Lithium regulates the *Cck* gene in part by causing an increase in H3K4me3 in *Clock*Δ*19* mice. The precise mechanism of this increase remains unclear, though recruitment of other complexes containing histone methyltransferases may be involved, as CLOCKΔ19 levels decrease at the *Cck* promoter following lithium treatment along with the increase in H3K4me3. These results will help identify more selective therapeutic targets for the development of novel mood stabilizing medications which may be more effective with fewer side effects than current treatments.

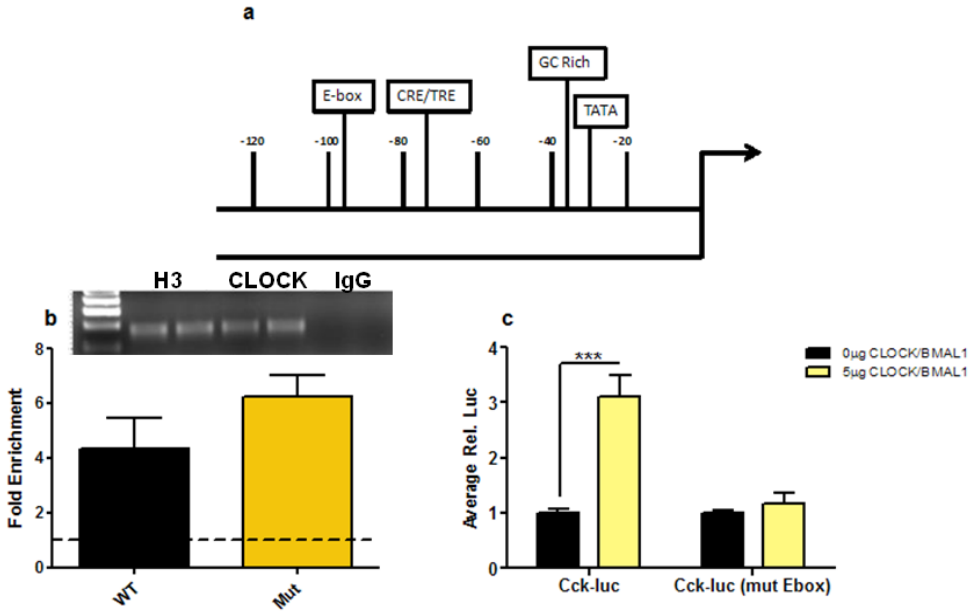


Figure 2-1. CCK is a CLOCK target gene and is regulated by the E-box element. A) Diagram of the cholecystokinin promoter. The region containing the proximal promoter region, including the E-Box, was amplified by quantitative qPCR after Chromatin immunoprecipitation (ChIP) assays were performed. Additional important transcription factor binding sites and regulatory regions are highlighted. **B)** Fold enrichment at proximal promoter region following ChIP with a CLOCK specific antibody comparing *Clock*Δ*19* mutants and wild-type (WT) littermate controls. One sample t-tests revealed that CLOCK is significantly enriched at the *Cck* promoter regions (~4-6 fold) above background in both WT (t_4=2.920, p = 0.0432) and *Clock*Δ*19* (Mut) (t_3=6.754. p = 0.0066) mice. Inset are representative agarose gels of q-PCR products from ChIP assays showing AcH3 positive control IPs, IgG negative control IPs, and CLOCK IPs; (n=4-6 per genotype). **C)** Relative luciferase activity of PC12 cells transfected with a Cck-luc construct (318 bp) containing either an intact or mutated E-box element. Co-transfection of 5µg of CL OCK and BMAL1 expression constructs resulted in a significant increase in Cck-luc activity (t_{14}=5.314, p =0.0001) when the E-box element was intact. Induction of Cck-luc activity was not detected when the E-box element was mutated; (n=5-8 per group).

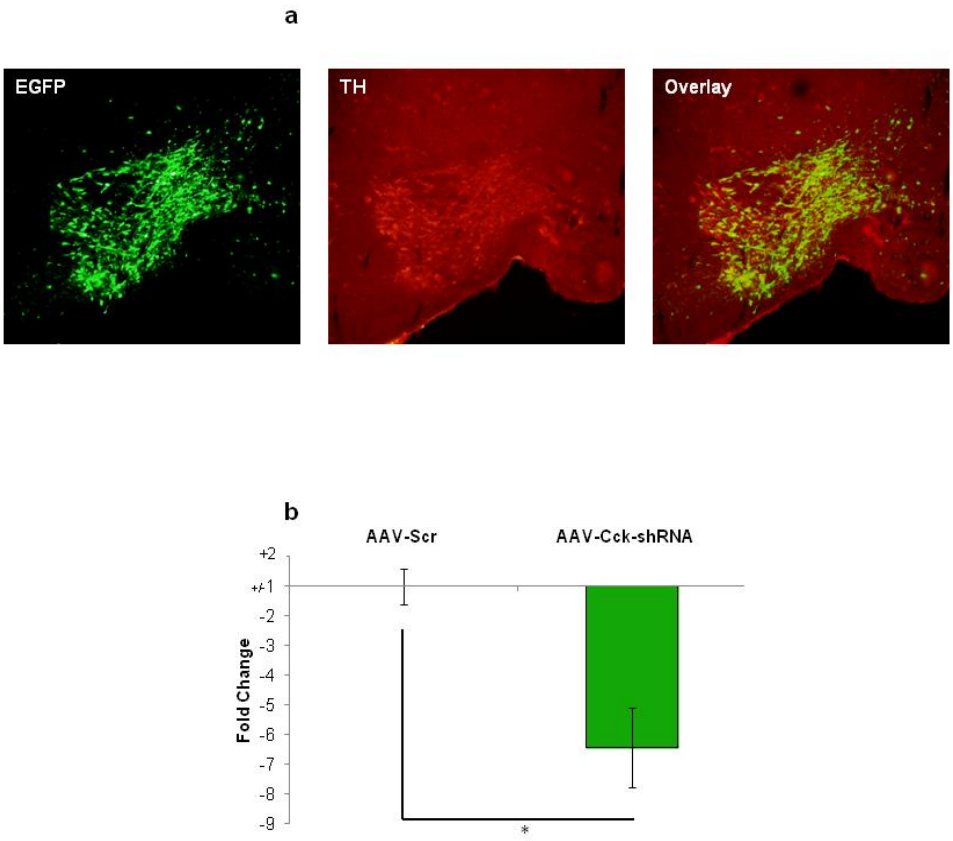


Figure 2-2. *In vivo* characterization of AAV-Cck-shRNA. A) Representative images showing VTA specific targeting of AAV-*Cck*-shRNA. AAV-expressing shRNA was stereotaxically injected into the VTA. Three weeks following surgery, brain sections were immunostained with anti-green fluorescent protein (Green) and anti-tyrosine hydroxylase (Red) antibodies and images were merged to see colocalization using an epifluorescence microscope. Shown is a 10x image. **B)** Injection AAV-Cck-shRNA results in a reduction of *Cck* mRNA in the VTA of mice. Three weeks following AAV-injection, brains were dissected and neurons expressing EGFP were collected using laser capture microscopy. RNA was then purified from isolated cells, cDNA was synthesized and subjected to quantitative PCR using *Cck*-specific primers. A significant reduction in Cck mRNA levels was observed in AAV-Cck-shRNA infected cells relative to AAV-Scr controls (t_4= 2.929, p = 0.04); (n= 3-4 animals per group

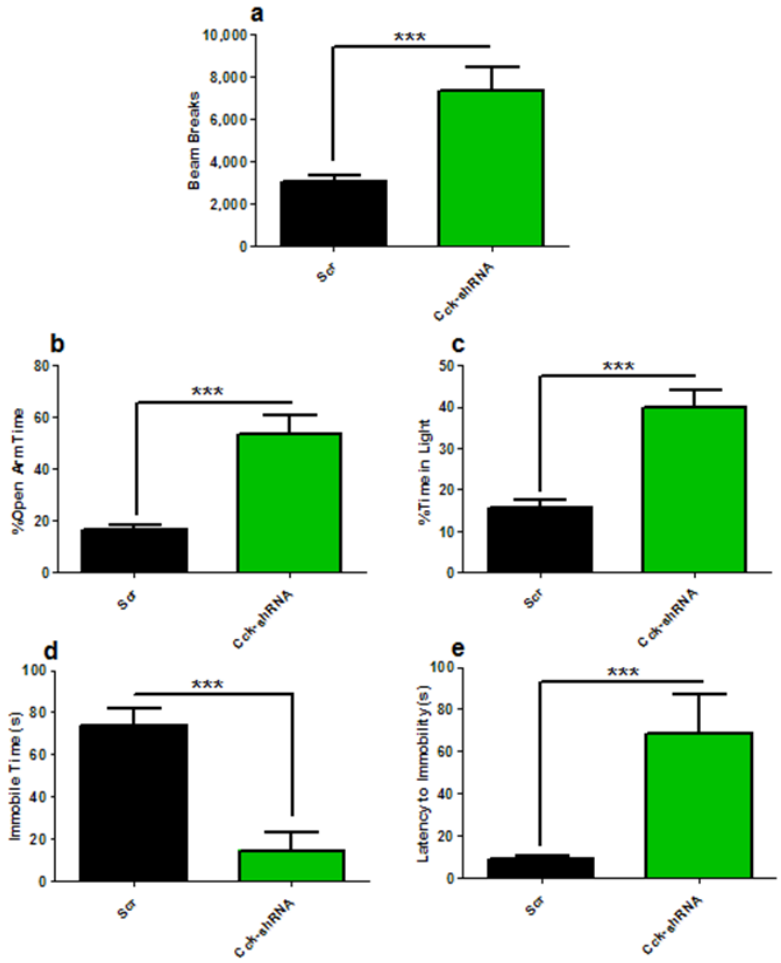


Figure 2-3. Knockdown of Cck in the Ventral Tegmental Area of Wild Type animals results in a manic-like phenotype. A) Locomotor activity of AAV-Cck-shRNA and AAV-Scr injected C57/BL6 animals was assessed for two hours 3 weeks after stereotaxic injection. AAV-Cck-shRNA injected animals are hyperactive when compared to AAV-Scr controls (t_{18}= 3.756, p =0.0014). **B-C)** Anxiety-related behavior was assessed in AAV-Cck-shRNA and AAV-Scr injected animals using the elevated plus maze (EPM,**B**) and dark/light box (**C**). AAV-Cck-shRNA injected animals are significantly less anxious than AAV-Scr controls as seen by an **B)** increase in open arm time on the EPM (t_{16}=4.90, p=0.0002) and **C)** time spent in the light side of the dark/light box (t_{20}=5.528, p<0.0001). **D-E)** Depression-related behavior was assessed in AAV-Cck-shRNA and AAV-Scr injected animals using the forced swim test. AAV-Cck-shRNA injected animals display less depression-related behavior than AAV-Scr controls as evidenced by **D)** a decrease in total immobile time (t_{32}=4.935, p<0.0001) and **E)** and increased latency to first bout of immobility (t_{14}=3.126, p=0.0074); (n=15-20 per group).

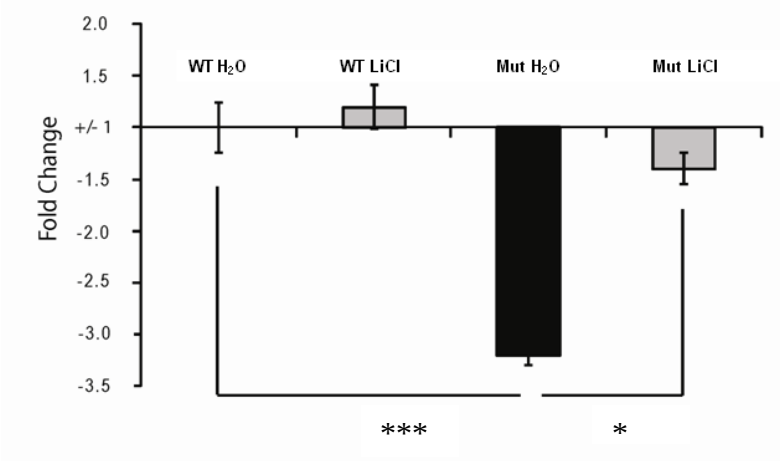


Figure 2-4. Lithium treatment selectively restores Cck mRNA levels in the VTA of *Clock*Δ19 mice. Relative mRNA levels of *Cck* in *Clock*Δ*19* (Mut) mice and WT littermates receiving 10 days of water or lithium treatment (Li, 600mg/L). Levels were normalized to an internal control, *Gapdh*. Analysis by two-way ANOVA revealed a significant decrease in *Cck* mRNA levels in untreated *Clock*Δ19 mice compared to WT animals (main effect of genotype $F_{1,20}=16.99$; $p=0.0005$). Bonferroni post hoc tests revealed that lithium treatment caused a significant increase in *Cck* expression in *Clock*Δ19animals, restoring it to near WT levels ($t=2.600$, $p<0.05$). Lithium treatment had no detectable effect of WT *Cck* expression.

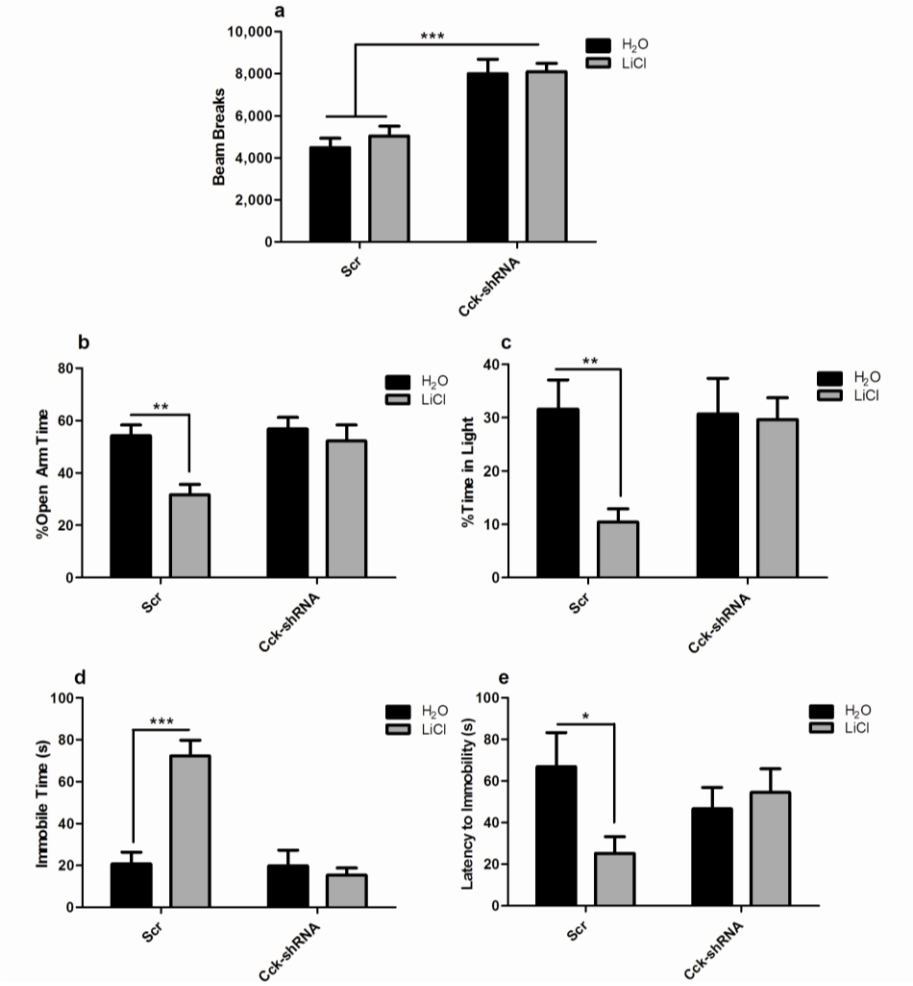


Figure 2-5. An increase in Cck levels in the VTA is required for lithium's therapeutic actions in the *Clock*Δ*19* mice A) Locomotor activity was measured in AAV-Cck-shRNA or AAV-Scr injected *Clock*Δ19 mice for two hours following 10 days of lithium (LiCl) administration. Analysis by two-way ANOVA revealed a main effect of viral injection on locomotor activity ($F_{1,53}$=36.34, p<0.0001). Bonferroni post-tests revealed that there was no significant effect of any treatment on locomotor response to novelty. **B-C)** Anxiety-related behavior was assessed in AAV-Cck-shRNA and AAV-Scr injected *Clock*Δ19 mice following lithium treatment using the EPM (**B**) and dark/light box (**C**). Analysis by two-way ANOVA followed by Bonferonni post-tests revealed that lithium treatment causes a significant increase in anxiety-related behavior in AAV-Scr injected animals as seen by **B)** a decrease in time spent in the open arms of the elevated plus maze (t=3.051, p <0.01 and **C)** a decrease in time spent in the light side of the dark/light box (t=3.343, p<0.01). Lithium treatment had no detectable effect on AAV-Cck-shRNA injected animals. **D-E)** Depression-related behavior following lithium treatment was assessed in AAV-Cck-shRNA and AAV-Scr injected *Clock*Δ19 animals using the forced swim test. Analysis by two-way ANOVA followed by Bonferonni post-tests revealed that lithium treatment causes a significant increase in depression-related behavior in AAV-Scr injected animals as seen by **D)** an increase in total immobile time (t=5.986, p<0.0001), and **E)** a decrease in latency to first bout of immobility (t=2.513, p<0.05). Lithium treatment had no detectable effect on AAV-Cck-shRNA injected animals; (n=12-20 per group).

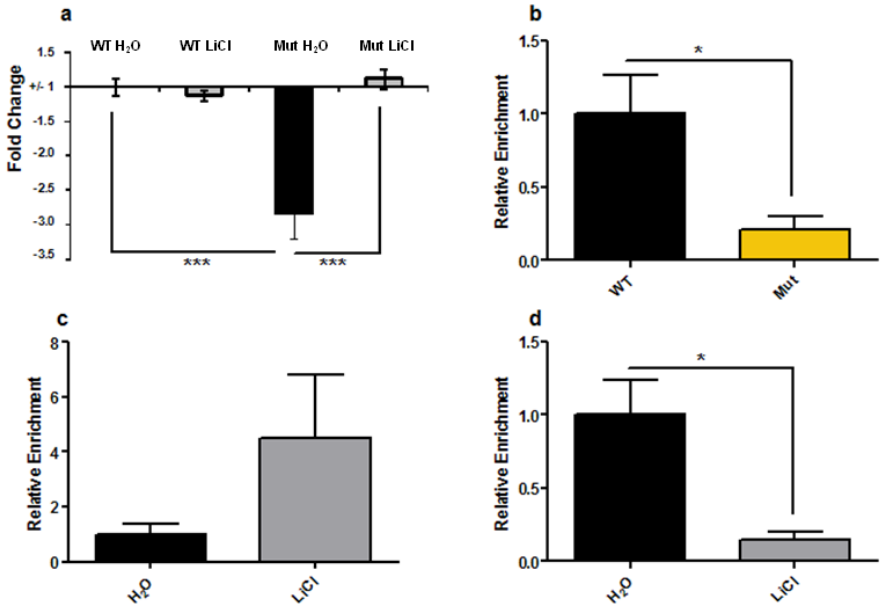


Figure 2-6. Effect of ClockΔ19 mutation and lithium treatment on H3k4me3 and MLL1 at the Cck promoter. **A)** Relative levels of histone H3K4me3 at the *Cck* promoter in *Clock*Δ*19* mice (Mut) and WT littermates following lithium (LiCl) treatment were assessed by performing ChIP assays with a H3K4me3 specific antibody. Analysis by two-way ANOVA revealed a main effect of genotype on H3K4me3 levels ($F_{1,18}$=16.80, p=0.0007). Bonferonni post-tests revealed that lithium treatment caused a significant increase in H3K4me3 levels in *Clock*Δ*19* mice (t=7.091, p<0.0001), but had no effect on WT animals; (n=5-6 per group). **B)** Relative enrichment of MLL1 at the *Cck* promoter in *Clock*Δ*19* mice and WT littermates was assessed by performing ChIP assays with an MLL1-specific antibody. A significant decrease in MLL1 at the *Cck* promoter was observed in *Clock*Δ*19* mice (t_8=2.827, p = 0.0223); (n= 5 per group). **C)** Relative enrichment of MLL1 at the *Cck* promoter in *Clock*Δ*19* mice following lithium treatment was assessed by performing ChIP assays with an MLL1-specific antibody. There was a non-significant trend towards increased MLL1 levels in lithium-treated *Clock*Δ*19* mice (t_8=1.865, p = 0.0992);(n=5-6 per group) **D)** Relative enrichment of CLOCK at the *Cck* promoter in *Clock*Δ*19* mice following lithium treatment was assessed by performing ChIP assays with a CLOCK-specific antibody. A significant decrease in CLOCK at the *Cck* promoter was observed in lithium-treated *Clock*Δ*19* mice (t_9=3.137,p=0.0120); (n=5-6 per group).

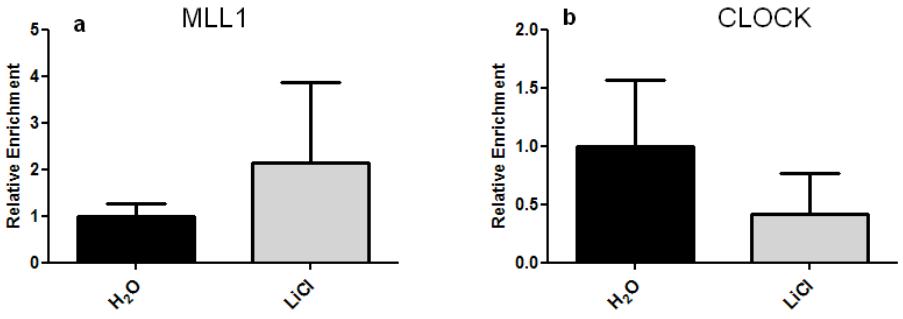


Figure 2-7. Effects of lithium treatment on MLL1 and CLOCK binding to the *Cck* promoter in WT animals. A) Relative enrichment of MLL1 at the *Cck* promoter in WT mice following lithium (LiCl) treatment was assessed by performing ChIP assays with an MLL1-specific antibody. There was a no detectable change in MLL1 at the *Cck* promoter following lithium treatment; (n=5-6 per group). **B)** Relative enrichment of CLOCK at the *Cck* promoter in WT mice following lithium treatment was assessed by performing ChIP assays with a CLOCK-specific antibody. There was a no detectable change in CLOCK at the *Cck* promoter following lithium treatment; (n=5per group).

CHAPTER THREE

REGULATION OF HISTONE ACETYLATION IS IMPORTANT FOR THE THERAPEUTIC ACTIONS OF PHARMACALOGICAL TREATMENTS FOR MANIC-LIKE BEHAVIORS

Introduction:

Bipolar disorder (BPD) is a severe and chronic psychiatric disease that is defined by the occurrence of one or more manic or mixed episodes (Kessler, Berglund et al. 2005). These manic or mixed episodes cycle with periods of depression, which is why BPD was once referred to as manic-depressive illness. Symptoms of mania include elevated mood, irritability, decreased need for sleep, hyperactivity, and impulsive behaviors (Miklowitz and Johnson 2006). The depressive phase of BPD resembles major depressive disorder, with dysphoria, anhedonia, and disrupted sleep patterns. Mixed states have characteristics of both mania and depression. These various mood states cycle with periods of euthymia, or normal mood (Miklowitz and Johnson 2006).

Bipolar mania and mixed states are treated with mood stabilizers, which are drugs that ameliorate manic symptoms without cause triggering a depressive episode (Miklowitz and Johnson 2006). Two of the most commonly used mood stabilizers in the treatment of BPD are lithium and valproate (VPA). Though these drugs have therapeutic effects in the treatment of BPD, there are several drawbacks to their use. Side effects of lithium treatment include sedation, stomach irritation, thirst, motor tremors, weight gain, hypothyroidism, and kidney clearance issues, which can lead to non-adherence and "rebound" manic episodes (Miklowitz and Johnson 2006; Vieta and Sanchez-Moreno

2008). An advantage of VPA treatment is that it has a milder side effect profile than lithium. VPA side effects include nausea, weight gain, stomach pain, fatigue, depressed platelet counts, and elevated liver enzymes (Miklowitz and Johnson 2006; Vieta and Sanchez-Moreno 2008). Because of these adverse side effects, and the fact that not all patients respond to current moods stabilizers, more effective treatments could be developed based upon the actions of drugs presently used to treat BPD.

Despite being in clinical use for many years, decades in the case of lithium, the molecular mechanisms of these mood stabilizers have yet to be elucidated. Proposed mechanisms of action for lithium include the altering neurotransmission of dopamine and serotonin (Treiser, Cascio et al. 1981; Baldessarini and Vogt 1988; Coque, Mukherjee et al. 2011). In addition to affecting neurotransmission, lithium can affect many intracellular signaling cascades. This is thought to be due to its competition with magnesium, which a co-factor for many proteins (Mota de Freitas, Castro et al. 2006). Molecules thought to be affected by lithium include the serine-threonine kinase glycogen synthase kinase - 3β (GSK-3β), which has over 30 identified targets in the cell that can affect many neuronal processes, including regulation of transcription, neuroprotection and apoptosis, circadian rhythms, neurite outgrowth, and cytoskeletal remodeling (Frame and Cohen 2001; Doble and Woodgett 2003; Iitaka, Miyazaki et al. 2005).

Lithium has also been hypothesized to act through inositol depletion, which can affect signaling via the second messengers inositol 1,4,5-tris-phosphate and diacylglycerol, which in turn can affect protein kinase C and Calmodulin-dependent protein kinase II, and intracellular calcium signaling (Gould, Quiroz et al. 2004; Harwood 2005; Schloesser, Huang et al. 2008). Lithium can also affect G-protein signaling via

magnesium competition, including G-proteins themselves, adenylyl cyclases, and arrestin complexes, which function downstream of G-proteins (Mota de Freitas, Castro et al. 2006). Additionally, lithium can lengthen the circadian period of many organisms (Klemfuss 1992). This was originally thought to be due to GSK-3β inhibition, but has recently become controversial (Iitaka, Miyazaki et al. 2005; Hirota, Lewis et al. 2008).

Similar to lithium, VPA is a small molecule that can affect a number of cellular signaling pathways. It can also affect neurotransmission, specifically the GABAergic and glutamatergic systems (Gurvich and Klein 2002). VPA also can affect some of the same cell signaling pathways as lithium treatment. VPA may also inhibit GSK-3β, but this finding has been controversial (Chen, Huang et al. 1999; Phiel, Zhang et al. 2001; Jin, Kovacs et al. 2005). Though it is uncertain whether or not VPA can inhibit GSK-3β like lithium, it has been shown to affect some of the downstream targets of GSK-3β in a manner similar to lithium, including enhancement of β-catenin dependent transcription (Gurvich and Klein 2002). VPA has also been shown to reduce inositol signaling, and lengthen the circadian rhythms of *Drosophila* (Dokucu, Yu et al. 2005; Terbach and Williams 2009).

VPA can also affect chromatin structure, as it has more recently been found to be a direct inhibitor of histone deacetylases (HDAC, (Phiel, Zhang et al. 2001). HDACs are a class of enzymes that remove acetyl groups from the N-terminal tails of histone proteins (Zhang and Meaney 2010). Through this mechanism they regulate chromatin conformation and the ability of transcriptional regulators to access DNA (Zhang and Meaney 2010). Acetylation is associated with gene activation, thus HDACs generally act as transcriptional repressors (Zhang and Meaney 2010). VPA inhibits both Class I and II

HDACs; therefore, it can potentially affect the expression of many genes through this action (Phiel, Zhang et al. 2001; Gurvich and Klein 2002). This activity of VPA may be important for its mood stabilizing actions, as it has recently be found that systemic administration of sodium butyrate (SB), another Class I and II HDAC inhibitor (HDACi) can reverse and prevent amphetamine induced hyperlocomotion in rats, which has been used to model mania (Moretti, Valvassori et al. 2011). However, SB has many non-HDACi activities, and it remains unclear which of these actions is responsible for the effects of SB on amphetamine induced hyperlocomotion (Moretti, Valvassori et al. 2011). Additionally, amphetamine induced hyperlocomotion has many caveats as an accurate model of mania, including that molecular and cellular changes in the brain following this paradigm in wild type (WT) animals may not be the same as those found in mania, which is a pre-existing disease state (Nestler and Hyman 2010). It therefore remains to be fully determined whether administration of an HDACi can result in mood stabilizer-like effects.

Studies from our own lab have found that chronic administration of the mood stabilizer lithium has therapeutic effects on the mood-related behavior of *Clock*Δ19 model of mania (Roybal, Theobold et al. 2007). These mice bear a point mutation in the *Clock* gene, a transcription factor that controls circadian rhythms, that results in a CLOCK protein (CLOCKΔ19) with a dominant negative function (Vitaterna, King et al. 1994). *Clock*Δ19 mice have a complete behavioral profile which is strikingly similar to human mania (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007). *Clock*Δ19 mice display hyperactivity, decreased anxiety-related and depression-related

behavior, and increased preference for rewarding stimuli (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007).

Lithium treatment restores anxiety and depression-related behaviors of *Clock*Δ19 mice to WT levels (Roybal, Theobold et al. 2007). Interestingly, this behavioral rescue is selective to *Clock*Δ19 mice, as lithium treatment either has no effect or an opposite effect on WT animal behavior. Most studies on lithium's mechanism of action have been performed in WT animals, which may be difficult to interpret because these mice model a "normal" human control, which does not respond to lithium treatment in the same manner as a BPD patient. The *Clock*Δ19 mice provide a unique opportunity to study lithium's mechanism of action, because lithium treatment does not have the same effects on healthy subjects as BPD patients, similar to what is observed in the *Clock*Δ19 mice (Malhi, Adams et al. 2009).

In the present study, we set out to determine if VPA treatment also has therapeutic effects in *Clock*Δ19 mice, similar to lithium, which would add further predictive validity to these mice as a model of mania. Because VPA and lithium can affect multiple pathways and myriad proteins within the cell, we hypothesized that their mood stabilizing actions may be a result of regulation of common target genes. We recently identified a lithium target gene, *cholecystokinin* (*Cck*), that is important for lithium's therapeutic actions in the *Clock*Δ19 mice (Chapter 2). We hypothesized that if VPA has anti-manic actions in the *Clock*Δ19 mice, that it may also regulate *Cck* in a manner similar to lithium. We also set out to determine whether or not VPA's activity as an HDACi could play a role in its mood stabilizing actions, both at the molecular level

and by assessing the effects of chronic administration of a specific Class I HDACi, suberoylanilide hydroxamic acid (SAHA), on *Clock*Δ19 behavior.

Materials and Methods

Animals and Housing *Clock*Δ*19* mutant mice were created by *N*-ethyl-*N*-nitrosurea mutagenesis and produce a dominant-negative CLOCK protein defective in transcriptional activation activity as described (King, Vitaterna et al. 1997). For all experiments using *Clock*Δ*19* mutants, 8 to 16 week old adult male mutant (*Clock*Δ*19;* Mut*)* and wild-type (WT) littermate controls on a mixed BALBc; C57BL/6 background were used. Mice were group housed in sets of 2-4 per cage on a 12:12 h light/dark cycle (lights on 6:00 a.m., lights off at 6:00 p.m) with food and water provided *ad libitum* All mouse experiments were performed in compliance with National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committees of UT Southwestern Medical Center.

Lithium Administration Lithium treated mice received 600mg/l of LiCl in drinking water for 10 days prior to behavioral testing, and throughout the course of the testing. This administration results in a stable serum concentration of lithium in the low therapeutic range for human patients (0.41±0.06 mmol/l), with little to no adverse health consequence (Roybal et al, 2007).

Valproic Acid Administration : After a one week habituation period, mice received 20 g/kg sodium valproate in their chow (Teklad animal diets, Harlan laboratories) for two

weeks before behavioral testing. Administration continued throughout the course of testing. This administration results in a stable serum concentration in the low therapeutic range for humans (50-80 μg/ml) with little to no adverse health consequences (Gould, Chen et al. 2004; Hao, Creson et al. 2004).

SAHA administration: For oral, chronic administration of the HDAC inhibitor SAHA, SAHA was complexed with cyclodextrins in drinking water in a manner similar to Hockly et al., 2003. In brief, 18 g of 2-hydroxypropyl-β-cyclodextrin (HOP-β-CD) was dissolved in water. 0.67 g of SAHA was added to the solution and heated until fully dissolved, and then cooled to room temperature. This solution was given to the mice in place of drinking water for two weeks. The solution was replaced at least weekly. Vehicle was 18g of HOP- β-CD in water without SAHA. This administration method has been shown to mimic the effects of daily injections of SAHA at concentrations of 100-200 mg/kg, which causes increases in histone acetylation (Hockly, Richon et al. 2003).

Chromatin Immunoprecipitation (ChIP): ChIP assays were performed according to methods described previously (Tsankova, Kumar et al. 2004; Enwright, Wald et al. 2010). Briefly, 1mm VTA containing midbrain sections taken from *Clock*Δ19 mice or WT littermates were cross-linked in 1% formaldehyde for 15 minutes. Cross-linking was then quenched by the addition of glycine at a final concentration of 0.125M for 5 minutes. Chromatin was sheared to 0.2 to 1kb fragments by sonication and cleared with Protein A beads (Thermo Scientific #22811). For each pull-down, approximately 60-100μg of chromatin was used. Chromatin was incubated, rotating overnight at 4°C, 5-10μg of one of the following antibodies: acetylated histone H3 (Millipore #06-599),

acetylated histone H4 (Millipore #06-866), and IgG (Millipore #12-370). The antibody-chromatin complexes were immunoprecipitated using Protein A beads plus according to manufacturer's instructions. Following reverse cross-linking of input and immunoprecipitated samples, quantitative PCR was performed to determine levels of protein binding at promoters of interest using primers against the proximal *Cck* promoter. Input DNA and immunoprecipitated DNA were amplified in duplicate in the presence of SYBR Green on the ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA). Relative quantification of template DNA was performed using the fold enrichment method.

Behavioral Assays

Locomotor Response to Novelty: Mice were placed into individual automated locomotor activity chambers that were equipped with infrared photobeams (San Diego Instruments, San Diego, CA). Activity measurements commenced upon the first beam break and were measured continuously and data was collected in five minute blocks over a period of two hours.

Elevated Plus Maze: The plus maze apparatus consisted of closed and open arms (all arms are 30 x 5 cm, with 25 cm tall walls on the closed arms). Mice were placed in the center of an elevated plus maze and the time spend in the open arms, closed arms, and center of the maze, along with the number of entries into the open and closed arms of the maze were determined by Ethovision 3.0 video tracking software (Noldus, Leesbrg, VA). Time spend on the open arm and percent of entries in to the open arm were used to determine anxiety-related behavior. The apparatus was cleaned and allowed to dry between every mouse.

Dark/Light Test: The dark/light apparatus is a 2 chambered box (25 cm x 26 cm for each side, Med Associates, St. Albans, VT), one side of which was kept dark, and the other side brightly lit by a fluorescent bulb at the top of the chamber. Mice were allowed to habituate to the dark side of the box for two minutes. Following the habituation period, the door between the compartments was opened and they were allowed to freely explore both sides of the apparatus for 10 minutes. Anxiety-like behavior was measured as the percent of time spent in the light side.

Forced Swim Test: Mice were placed in 4 liter Pyrex glass beakers were filled with 3 liters of water at 21-25°C for 6 minutes. All test sessions were recorded by a video camera from the side of the beakers. Water was changed between subjects. The video was analyzed and scored by an observer blind to the genotypes and treatment groups. After a 2 minute habituation time, latency to immobility was determined as the first cessation of movement. Total immobility was measured during the last four minutes of the test and was measured as time spent without movement except for a single limb paddling to maintain flotation.

Learned Helplessness: For initial training, mice were placed in a two sided shuttle chamber (Med Associates) with a stainless steel grid floor and automatic guillotine door, where they received foot shocks (5 second duration, 0.35 mA) per minute at for one hour, with the door of the chamber closed so that the mouse could not escape. This one hour training was repeated for two days. On the third day, the guillotine door was opened upon the delivery of each foot shock (0.35 mA, 25 seconds), allowing the mouse to escaped to the other side of the chamber. Fifteen trials were received on the test day. The latency to escape and the number of failures to escape was measured.

Quantitative PCR: cDNA or purified genomic DNA was mixed with buffers, primers, SYBR green, and hot start Taq polymerase in a master mix prepared by a manufacturer (Applied Biosystems, Foster City, CA). Using a Real-Time PCR machine (7500 Real Time PCR machine, Applied Biosystems) PCR reactions were run followed by a dissociation reaction to determine specificity of the amplified product. The amount of gene expression was quantified using the ΔΔCt method as previously described.

Western Blotting: Histones were isolated for immunoblotting as in Fischer et al., 2007. Brain tissue was homogenized in TX-buffer (50mM Tris HCl, 150mM NaCl, 2mM EDTA, 1% Triton-100) and incubated on ice for 15 minutes. The samples were then centrifuged at 2,000 r.p.m. at 4°C for 10 minutes. The samples were then washed in TX buffer. The pellet was then dissolved in TX buffer containing 0.2 M HCl and incubated on ice for 30 minutes followed by centrifugation at 10,000 r.p.m. at 4°C for 10 minutes. The supernatant was isolated and then used directly for immunoblotting. Aliquots of sample were combined 1:1 in Laemmli SDS sample buffer (Bio-World, Dublin, OH), and heated at 95°C for 5 minutes. Samples were loaded and electrophoresed on a pre-cast 12% Tris-glycine extended gel (Biorad, Hercules, CA) at 120V for ~ 90 minutes in 1XTGS buffer (Biorad, Hercules, CA). Proteins were transferred at 90V at 4°C for 45 minutes onto Immobilon PVDF membranes (Millipore, Bedford, MA) 1XTG buffer. Membranes were re-wet briefly in a series of methanol, MilliQ water and 1XTBS and then blocked in 5% non-fat dry milk dissolved in 1xTBS + 0.1% Tween20. for 1 h at room temperature (RT). Membranes were incubated overnight at RT with the following primary antibodies diluted in 5% non-fat dry milk dissolved in 1xTBS + 0.1% Tween20: acetylated histone H3 (Millipore #06-599), acetylated histone H4 (Millipore #06-866),

total histone H3 (Millipore #07-690,) , total histone H4 (Millipore #04-858). Blots were stripped (625mM Tris pH 6.8, 2% SDS, 700µl β-mercaptoethanol per 100 ml) following application of the acetylated histone and reprobed with total histone antibodies. Blots were washed in 1XTBS + 0.1% Tween20 and incubated for 1 hour at RT with secondary antibodies (Anti-Rabbit, Vector #PI-1000). Blots were washed in 1XTBS + 0.1% Tween20 with a final wash in 1XTBS. Scion Image (Frederick, MD, USA) was used to assign absolute immunoreactivity to the bands, and a scanner was used to take digital images of the films. These values were then expressed as a ratio of acetylated histones to total histones.

Primer Sequences: *Gapdh*: Forward – 5'-AACGACCCCTTCATTGAC-3', Reverse- 5'-TCCACGACATACTCAGCAC-3'

Cck: Forward- 5'-ACTGCTAGCGCGATACATCC-3' , Reverse- 5'-TTCGTAGTCCTCGGCACTGC-3'

Cck Promoter: Forward- 5'-CTTGGGCTAGCCTCATTCACTG-3', Reverse- 5'-TTAAATAGCTCCTCCCGGTTCG-3'

Human Gapdh: Forward - 5'-ATGGGGAAGGTGAAGGTCG-3', Reverse - 5'-GGGGTCATTGATGGCAACAATA-3

Human Cck: Forward – 5'-CGTAGGCAGCTGAGGGTATCGCA-3', Reverse- 5'-TATCCTGTGGCTGGGGTCCAGG-3'

Statistical analysis

For the comparison of two groups, two-tailed unpaired Student's t tests were used, and one-way ANOVAs followed by Bonferroni post tests for multiple comparisons were performed for the comparison of three groups. When more than one factor was examined

simultaneously, two-way ANOVAs were performed followed by Bonferroni posttests. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

VPA has therapeutic effects in *Clock*Δ19 mice

To determine the effects of VPA administration on manic-like behaviors, *Clock*Δ19 mice received VPA in an oral, chronic manner. Mice received either VPA at 20g/kg in their chow or regular chow (RC) that had been processed in the same manner as VPA chow, for two weeks. This administration was followed by a battery of behavioral testing. Mice were first assessed for their locomotor response to novelty. As reported previously (Roybal, Theobold et al. 2007), *Clock*Δ19 mice are hyperactive when compared to WT animals (Figure 3-1a). VPA treatment had no significant effect on locomotor activity, similar to mice receiving lithium treatment (Figure 3-1a). Because locomotor activity was unaffected by treatment, we can feel confident that the effects on the other behavioral tests are due exclusively to changes in anxiety and depression related behavior and are not the result of changes in general activity.

To examine the effects of VPA administration on anxiety-related behavior, mice were subjected to two different measures: the elevated plus maze and dark/light test. In the elevated plus maze, VPA rescued the increased exploratory behavior of *Clock*Δ19 mice as seen by a reduction in the amount of time spent in the open arms of the elevated plus maze, similar to lithium treatment (Figure 3-1b,(Roybal, Theobold et al. 2007)). VPA treatment also had anxiogenic effects in the dark/light test similar to lithium treatment, reducing the time *Clock*Δ19 mice spent on the light side (Figure 3-1c,(Roybal,

Theobold et al. 2007)). There was no detectable effect of VPA treatment on WT animals in any measure of anxiety-related behavior (Figure 3-1b,c), which is also similar to the effects of lithium treatment (Roybal, Theobold et al. 2007).

Mice were subjected to either the Porsolt forced swim test (FST) or the learned helplessness paradigm to examine the effects of VPA administration on depression-related behaviors. As described previously, *Clock*Δ19 mice spent significantly less time immobile in the FST, and failed to escape fewer times in the learned helplessness test, indicating a decrease in depression related behavior (Figure 3-1d,e; (Roybal, Theobold et al. 2007)). VPA caused a significant increase in immobile time in the FST and number of failures in the learned helplessness test in *Clock*Δ19 mice, restoring depression related behavior to near WT levels (Figure 3-1d,e). Similar to lithium treatment, WT animals showed no detectable changes in either measure of depression related behavior as a result of VPA treatment (Figure 3-1d,e;(Roybal, Theobold et al. 2007).

VPA and other BPD treatments increase *Cck* levels in the VTA

VPA has therapeutic effects on manic-like behaviors in the *Clock*Δ19 mice, similar to lithium treatment (Roybal, Theobold et al. 2007). We have recently identified a lithium target gene, *Cck* (Chapter 2). An increase in *Cck* in the ventral tegmental area (VTA) has been found to be necessary for lithium's therapeutic actions in the *Clock*Δ19 mice (Figure 2-5). We therefore set out to determine if VPA could also regulate the *Cck* gene. RNA was isolated from the VTA of *Clock*Δ19 and WT mice receiving either VPA or RC and qPCR was performed to assess *Cck* mRNA levels. As reported previously, *Cck* levels were decreased in the VTA of control *Clock*Δ19 mice relative to WT littermates (Figure 3-2a; (McClung, Sidiropoulou et al. 2005). *Cck* mRNA levels were

restored to near WT following VPA treatment (Figure 3-2a). WT *Cck* mRNA levels were also unaffected by VPA treatment, as was observed with lithium (Figure 3-2a, Figure 2-4).

Because a restoration of *Cck* levels in the VTA seems to correlate with the therapeutic actions of mood stabilizers in the *Clock*Δ19 mice, we set out to determine if *Cck* levels were also regulated by drugs that treat BPD in human postmortem tissue. RNA was isolated from the VTA of healthy control and BPD subjects, which were categorized as either on or off medication. Information on individual human postmortem tissue is detailed in Tables 3-1,3-2, and 3-3 . qPCR was performed to assess *Cck* mRNA levels. Unlike the *Clock*Δ19 mice, there was no detectable difference in *Cck* levels in the VTA between control subjects and BPD patients not receiving medication (Figure 3-2b). Interestingly, BPD patients receiving pharmacological treatment had a significant increase in *Cck* levels in the VTA (Figure 3-2b). This result suggests that an increase in *Cck* in the VTA may be a common effect of current treatments for BPD.

Regulation of histone acetylation by mood stabilizer treatment

Because we were able to establish that lithium and VPA have similar therapeutic effects on *Clock*Δ19 behavior, and that they can have similar effects on levels of the lithium target gene, *Cck*, we set out to determine what molecules may be regulated by these two mood stabilizers that leads to similar effects on gene expression. A previous study found that systemic administration of the nonselective Class I and II HDACi sodium butyrate mimics the effects of lithium and VPA in the amphetamine induced hyperlocomotion model of mania (Moretti, Valvassori et al. 2011). VPA is also a

nonselective HDACi, suggesting regulation of histone acetylation may be important for VPA's therapeutic actions, and potentially for the actions of other mood stabilizers.

We first set out to determine the effects of mood stabilizers on levels of general histone acetylation in the brain. Western blotting was performed on histones extracted VTA-containing midbrain tissue from *Clock*Δ19 or WT mice receiving lithium or VPA treatment, and their respective controls. Levels of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) were assessed relative to levels of total histones. Lithium treatment had no significant effect on levels of acetylated histones (Figure 3-3a,b). This result is not surprising as there is no report of lithium being able to inhibit HDACs on a global level. There was a significant increase in levels of both AcH3 and AcH4 in VPA treated animals compared to those receiving RC (Figure 3-3c,d), indicating that it is functioning as an HDACi in this treatment paradigm.

Because we had found that VPA can be functioning as an HDACi, we determined whether or not this was a potential mechanism by which VPA regulates the *Cck* gene. We also set out to determine whether or not lithium could regulate histone acetylation at the *Cck* promoter, because lithium has been shown to regulate trimethylation at histone H3K4 (H3K4me3, Chapter 2). A great deal of crosstalk has been shown between H3K4me3 and histone acetylation, as histone acetylation can stimulate methyltransferases that deposit this modification (Nightingale, Gendreizig et al. 2007). To assess levels of histone acetylation at the *Cck* promoter, ChIP assays were carried out using antibodies against AcH3 and AcH4 in *Clock*Δ19 mice and WT controls receiving either water or lithium (Figure 3-4a,b), or VPA or RC (Figure 3-4c,d), followed by qPCR with primers directed against the *Cck* promoter. Lithium treatment caused a

selective increase in levels of both AcH3 and AcH4 at the *Cck* promoter in *Clock*Δ19 mice, while WT levels of AcH3 and AcH4 are not significantly affected (Figure 3-4a-b). This selective increase is similar to what was observed previously with the H3K4me3 modification (Figure2-6a). In VPA treated *Clock*Δ19 mice, there was a robust increase in both AcH3 and AcH4 levels at the *Cck* promoter (Figure 3-4c,d). There was also a significant increase in AcH4 levels at the *Cck* promoter in WT animals, as well as a non-significant increase in AcH3 levels. This suggests that VPA is causing this increase via HDAC inhibition since it causes similar increases in overall histone acetylation in WT and *Clock*Δ19 animals (Figure 3-3c,d).

Administration of a Class I specific HDAC inhibitor to *Clock*Δ19 mice mimics the behavioral effects of VPA treatment

Because VPA acted as an HDACi in the *Clock*Δ19 mice, and appeared to regulate the *Cck* gene, which is known to be important for lithium's therapeutic effects, via HDAC inhibition, we hypothesized that VPA may rescue *Clock*Δ19 behavior by this activity. However, VPA can affect many other signaling pathways within the cell (Mitchell and Malhi 2002; Gould, Chen et al. 2004; Hao, Creson et al. 2004; D'Souza, Onem et al. 2009), so these pathways may also be responsible for VPA's mood stabilizing behaviors. To test this hypothesis, we administered the HDACi SAHA to *Clock*Δ19 mice in an oral, chronic manner as has been described previously (Hockly, Richon et al. 2003). Unlike VPA, SAHA targets only Class I and Class IIb HDACs. Furthermore, we chose to study the effects of SAHA on *Clock*Δ19 behavior because it has already been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (Lane and Chabner 2009; Bradner, West et al. 2010;

Bantscheff, Hopf et al. 2011; Inks, Josey et al. 2011). *Clock*Δ19 mice and WT animals received either SAHA complexed with a HOP-β-CD vehicle or HOP-β-CD vehicle dissolved in their drinking water for 2 weeks before being subjected to a battery of behavioral tests. Vehicle treatment did not cause any significant change in *Clock*Δ19 behavior as they displayed hyperactivity, decreased anxiety and depression related behavior when compared to vehicle treated WT animals (Figure 3-5).

Mice were first assessed for their locomotor activity. Similar to VPA and lithium, SAHA treatment had no significant effect on locomotor activity (Figure 3-5a; (Roybal, Theobold et al. 2007)) demonstrating that it is not a confounding factor in subsequent tests. The mice were then subjected the elevated plus maze and dark/light test to measure anxiety related behavior. In the elevated plus maze, SAHA had anxiogenic effects on the *Clock*Δ19 mice in both the elevated plus maze and dark/light test, causing a decrease in open arm time and decreased time in the light, respectively (Figure 3-5b,c). SAHA treatment had an opposite effect on WT anxiety related behavior as seen a non-significant increase in open arm time in the elevated plus maze and a significant increase in time spent in the light side of the dark/light test (Figure 3-5b,c). To examine the effects of SAHA administration on depression-related behavior, mice were then subjected to the FST. The decreased depression-related behavior of the *Clock*Δ19 mice was rescued by SAHA treatment, as it caused and increase in immobile time in the FST to near WT levels (Figure 3-5d). WT animals showed no detectable changes in immobile time, similar to VPA treatment (Figure 3-5d).

Molecular effects of SAHA administration in the *Clock*Δ19 mice

Once we had established that SAHA could rescue the manic-like behavior of the *Clock*Δ19 mice, we examined the molecular changes that occurred as a result of this treatment. We first verified that the behavioral effects of SAHA administration could be due to inhibition of HDACs in the brain. Western blotting was performed on histones extracted VTA-containing midbrain tissue from *Clock*Δ19 or WT mice receiving SAHA or HOP-β-CD vehicle. Levels of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) were assessed relative to levels of total histones. There was a significant increase in levels of AcH3 and a near significant increase in AcH4 in SAHA treated animals compared to those receiving HOP-β-CD vehicle (Figure 3-6a,b), indicating that it is indeed inhibiting HDACs in the brain, similar to what has been reported previously (Hockly, Richon et al. 2003).

We next examined whether SAHA administration had any effects on *Cck* levels in the VTA of *Clock*Δ19 mice, because it was found to be regulated by both lithium and VPA, and is necessary for lithium's therapeutic actions. Since SAHA also had rescued manic-like behaviors in the *Clock*Δ19 mice, we hypothesized that *Cck* levels in the VTA may be increased due to SAHA administration. RNA was isolated from the VTA of *Clock*Δ19 and WT mice receiving either SAHA or HOP-β-CD vehicle and qPCR was performed to assess *Cck* mRNA levels. As reported previously, *Cck* levels were decreased in *Clock*Δ19 mice receiving HOP-β-CD vehicle when compared to WT littermates (Figure 3-6c, (McClung, Sidiropoulou et al. 2005)). SAHA treatment had no detectable effect on *Cck* mRNA levels in the *Clock*Δ19 mice (Figure 3-6c). This indicates that SAHA rescues *Clock*Δ19 manic-like behavior by affecting genes other than

Cck, and suggests that other genes may be important for VPA's therapeutic effects as well. Interestingly, SAHA treatment caused a decrease in levels of *Cck* in the VTA of WT animals. This decrease could partially explain the anxiolytic effects that SAHA treatment has on WT animals, as we have shown that decreasing *Cck* in the VTA of WT animals causes decreased anxiety related behavior (Figure 2-3).

Discussion

Our results show that the mood stabilizer VPA has therapeutic effects on the manic-like behavior of *Clock*Δ19 mice, similar to what has been previously shown with lithium treatment (Figure 3-1(Roybal, Theobold et al. 2007). This adds further predictive validity to the *Clock*Δ19 mice as a model of mania, and suggests that they can be very useful in the screening of future compounds for the treatment of mania. Additionally, they can be studied to identify potential common targets of mood stabilizers.

Here we found that VPA regulates the *Cck* gene in the VTA of *Clock*Δ19 mice in a manner similar to what had been observed following lithium treatment, restoring decreased *Cck* mRNA levels to near WT (Figure 3-2a, 2-4). Like lithium treatment this increase is selective to the *Clock*Δ19 mice, and correlates with the selective regulation of *Clock*Δ19 behavior observed following VPA administration (Figures 3-1,3-2a). This result suggested that *Cck* in the VTA may be a common target gene for mood stabilizers. When we examined *Cck* mRNA levels in the postmortem VTA tissue from BPD patients, we found that unlike *Clock*Δ19 mice, there was no detectable difference between normal controls and BPD patients not receiving medications (Figure 3-2b), which corresponds

with untreated *Clock*Δ19 mice; however, there were very few patients in this group with a great deal of variability in *Cck* levels. It is also possible that a decrease in *Cck* may be only evident in a manic state, which is more similar to the phenotype of the *Clock*Δ19 mice, and the mood state of the BPD patients at the time of tissue collection was not known. Interestingly, *Cck* mRNA levels are significantly increased in the VTA of BPD patients that were receiving medications (Figure 3-2b). This is somewhat similar to what is observed in the *Clock*Δ19 mice, which had increases in *Cck* levels in the VTA following both lithium and VPA treatment (Figure 2-4, 3-2b), though the increase in BPD patients on medication was approximately 3-fold higher than normal controls, while *Clock*Δ19 mice had a restoration to near WT levels (Figure 3-2). It remains unclear whether or not this increase in *Cck* in the VTA of BPD patients following pharmacological treatment is important for therapeutic effects of medications. Even if this increase is not important for the function of mood stabilizers, it is still remarkable that BPD patients receiving a number of different pharmacological treatments in different combinations (Table 3-3) displayed a similar increase in *Cck* levels in the VTA. It is possible that this increase can be a marker of drug action for current treatments for BPD.

We also identified a role for the regulation of histone acetylation at the *Cck* gene in the *Clock*Δ19 mice by both lithium and VPA. Lithium treatment causes a selective increase in both AcH3 and AcH4 at the *Cck* promoter in *Clock*Δ19 mice (Figure 3-4a,b). As expected, this was not due to causing a general increase in histone acetylation throughout the brain (Figure 3-3a,b). This suggests that specific factors that mediate histone acetylation are being recruited to the *Cck* promoter following lithium treatment. This increase in histone acetylation is not surprising based upon the finding that lithium

causes a selective increase in H3K4me3 at the *Cck* promoter in *Clock*Δ19 mice (Figure 2-6a). It has recently become clear that H3K4me3 and histone acetylation are linked, and there is a great deal of crosstalk between proteins that deposit these modifications on histone tails (Martin and Zhang 2005; Nightingale, Gendreizig et al. 2007; Crump, Hazzalin et al. 2011; Guillemette, Drogaris et al. 2011). There is a great deal of overlap between histone acetylation and H3K4me3 at promoters, and it has been found that histone acetyltransferases (HATs) preferentially acetylate promoters that are marked by H3K4me3 and some HAT complexes may actually recognize H3K4me3, which may be how they are targeted specifically to promoters bearing this mark (Martin and Zhang 2005; Crump, Hazzalin et al. 2011; Guillemette, Drogaris et al. 2011). Conversely, it has also been shown that the activity of a histone methyltransferase that deposits H3K4me3 is stimulated by histone acetylation (Nightingale, Gendreizig et al. 2007). Thus the changes in histone acetylation at the *Cck* promoter in *Clock*Δ19 mice following lithium treatment are likely linked to the changes in H3K4me3, though it remains unclear which of these modifications is regulating the deposition of the other. Interestingly there is no decrease in levels of AcH3 or AcH4 at the *Cck* promoter in untreated *Clock*Δ19 mice, despite the previously observed decrease in H3K4me3 (Figure 2-6a, 3-4). This is probably due to the fact that CLOCKΔ19, though unable to recruit MLL1, still retains its HAT activity (Doi, Hirayama et al. 2006; Zhao, Malinin et al. 2007; Katada and Sassone-Corsi 2010).

VPA also regulates histone acetylation at the *Cck* promoter, causing increase in levels of AcH3 and AcH4 in *Clock*Δ19 mice (Figure 3-4c,d). Unlike lithium, this increase is not completely selective to the *Clock*Δ19 mice, as WT animals show a significant increase in levels of AcH4 and a trend towards an increase in levels of AcH3

at the *Cck* promoter following lithium treatment (Figure 3-4c,d). This result suggests that VPA may be regulating the *Cck* promoter through inhibition of HDACs, as VPA treatment results in an increase in overall levels of acetylated histones in both WT and *Clock*Δ19 mice (Figure 3-3c,d). Interestingly, this non-selective increase in histone acetylation at the *Cck* promoter does not result in a non-selective increase in *Cck* gene expression, as only *Clock*Δ19 mice have increases in *Cck* levels following VPA administration (Figure 3-2a). One possibility for this is that the *Cck* gene is already transcriptionally active in WT animals, and an increase in histone acetylation may not cause a further increase in transcription. This result also suggests that VPA treatment may allow for the *Cck* gene to be in a more transcriptionally permissive state in *Clock*Δ19 mice, and that other factors are responsible for the increased levels of *Cck* mRNA in the *Clock*Δ19 animals.

We were also able to determine that inhibition of HDACs may be important for VPA's mood stabilizing actions, as administration of a specific HDACi, SAHA, that is already in clinical use rescued the manic-like behaviors of *Clock*Δ19 mice. The importance of HDAC inhibition has already been suggested by other groups, because another HDACi, sodium butyrate was able to prevent amphetamine induced hyperlocomotion in rats. (Moretti, Valvassori et al. 2011). However, sodium butyrate has non-HDACi activities and amphetamine induced hyperlocomotion only models one aspect of mania, namely hyperactivity. Additionally this model is pharmacologically induced and transient, which makes it a less than ideal model of a disease state. Our results display that inhibition of HDACs alone, more specifically Class I and IIb HDACs, is sufficient to reverse manic-like behaviors in a genetic model of mania (Figure 3-5).

This finding suggests that specific HDAC inhibitors could be developed for use as future mood stabilizing agents.

When the molecular effects of SAHA treatment were examined, we determined that it was able to cause significant changes in levels of histone acetylation in the brain (Figure 3-6a,b). This suggests that its mood stabilizing effects are due to altering gene expression in the *Clock*Δ19 mice. However, when we examined the effects of SAHA administration on *Cck* expression in the VTA, which has been found to be increased following both lithium and VPA treatment, we found that there were no detectable changes in *Cck* mRNA levels. This result indicates that SAHA affects genes other than *Cck* to rescue *Clock*Δ19 behavior, and that an increase in *Cck* levels may contribute to the mood stabilizing actions of VPA in the *Clock*Δ19 mice, but may not be necessary for its therapeutic effects. It is likely that a dampening of dopaminergic transmission ultimately underlies the therapeutic effects of both VPA and SAHA, and a number of different proteins could lead to a similar effect on dopaminergic activity. A reason for this difference in *Cck* regulation could be that VPA inhibits both Class I and II HDACs, while SAHA only inhibits Class I and IIb HDACs (Phiel, Zhang et al. 2001; Bradner, West et al. 2010; Bantscheff, Hopf et al. 2011; Inks, Josey et al. 2011). VPA could be regulating the *Cck* gene via the inhibition of ClassIIa HDACs. If *Cck* is regulated by Class IIa HDACs, but not Class I or IIb HDACs, this could explain how VPA is causing an increase in acetylated histones at the *Cck* promoter and *Cck* gene expression, while SAHA has no detectable effect on *Cck* levels in *Clock*Δ19 mice. Interestingly, SAHA treatment leads to a decrease in *Cck* levels in the VTA of WT animals. The ability of SAHA to inhibit gene transcription has been reported previously in cell cultures by

causing the dissociation of complexes that activate transcription, and a similar effect may be occurring in WT animals (Chou, Wu et al. 2011). This decrease in *Cck* also correlates well with the anxiolytic effects of SAHA in WT animals, because a decrease in *Cck* in the VTA of WT animals has also been shown to be anxiolytic (Figure 2-3).

In conclusion we have found that the mood stabilizer VPA has therapeutic effects in the *Clock*Δ19 model of mania, similar to lithium. These actions correlate with an increase in *Cck* mRNA in the VTA of *Clock*Δ19 mice, suggesting that this gene may be a common target of mood stabilizers. We also found that treatments for BPD caused a significant increase in *Cck* mRNA levels in the VTA of human patients, indicating that an increase can possibly be a marker of drug action for current treatments for BPD. We found that both lithium and VPA regulate the *Cck* gene in *Clock*Δ19 mice by causing increases in histone acetylation in the *Cck* promoter. The increase in histone acetylation following lithium treatment is specific to the *Clock*Δ19 mice, and may be linked to previously observed changes in levels of H3K4me3. VPA causes increases in histone acetylation at the *Cck* promoter in both WT and *Clock*Δ19 mice, suggesting that this effect is due to HDAC inhibition. This finding that mood stabilizers can affect chromatin structure is not altogether surprising. Because these drugs require a chronic administration before having therapeutic efficacy, long lasting changes in neuroplasticity probably underlie their actions. Chromatin modifications are one way to mediate these changes, and other pharmacological treatments for psychiatric disease, such as antidepressants, exert their therapeutic effects via regulating chromatin remodeling enzymes (Tsankova, Berton et al. 2006; Covington, Maze et al. 2009). Therefore, mood stabilizers could be having similar effects on chromatin structure.

The role of chromatin remodeling in the therapeutic actions of VPA was further strengthened by the finding that administration of the specific Class I and IIb HDACi SAHA to *Clock*Δ19 mice mimicked the behavioral effects of VPA treatment, suggesting that inhibition of HDACs is important for VPAs therapeutic mechanism of action. SAHA administration rescued manic-like behaviors of *Clock*Δ19 mice without affecting *Cck* expression, suggesting that other genes are involved in the actions of SAHA. Thus alterations in *Cck* may be sufficient to treat a manic-like phenotype (as shown in chapter 2) but they are not necessary.

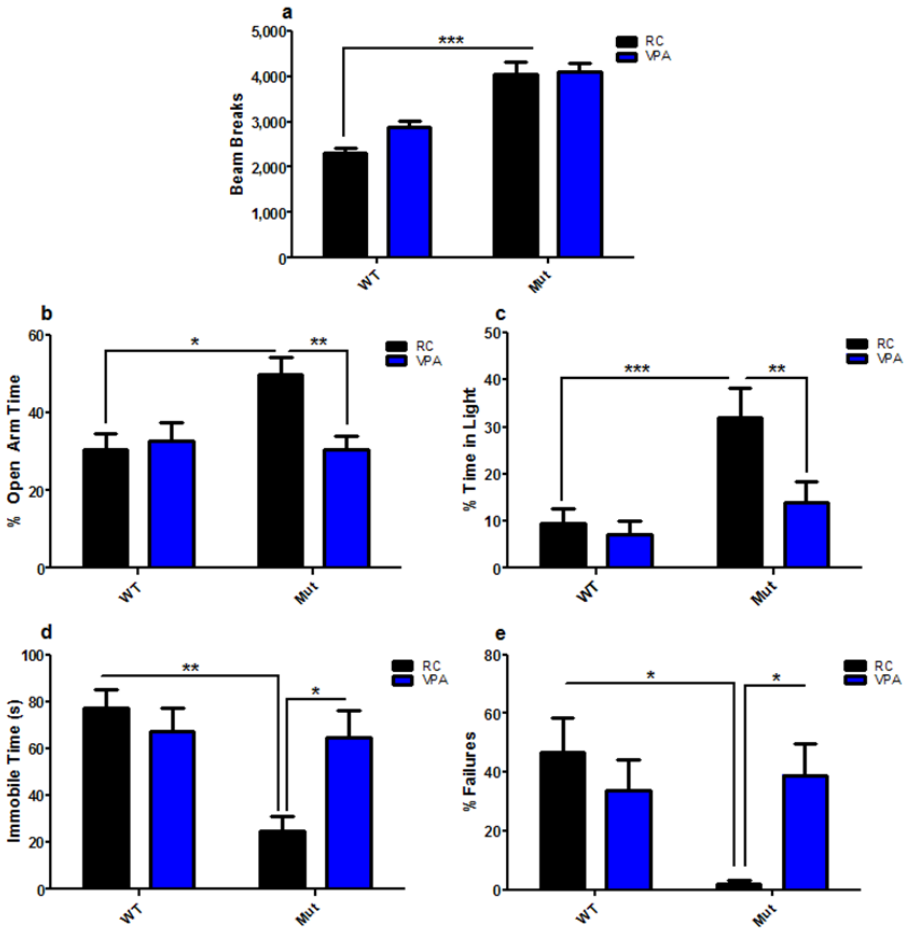


Figure 3-1. The mood stabilizer VPA has therapeutic effects in the *Clock*Δ19 mice. A) Locomotor activity was measured in *Clock*Δ19 (Mut) mice and wild type littermates for two hours following 2 weeks of VPA in chow or regular chow (RC). Analysis by two-way ANOVA revealed a significant main effect of genotype ($F_{(1,60)} = 67.11$, $p < 0.0001$). Bonferroni post-tests revealed that there was no significant effect of any treatment on locomotor response to novelty. **B-C)** Anxiety related behavior following VPA administration was assessed in both the elevated plus maze (EPM,**B**) and dark/light test (**C**). Analysis by two-way ANOVA revealed that *Clock*Δ19 mice spend more time in the open arms of the EPM (Main effect of genotype; $F_{(1,49)} = 4.24$, $p = 0.0447$) and light side of the dark/light box (Main effect of genotype; $F_{(1,55)} = 12.12$, $p = 0.0010$). Bonferroni post tests revealed that VPA treatment caused a significant reduction in open arm time in the EPM (**B,** $t = 3.213$, $p<0.01$) and in time spent in the light side of the dark/light test (**C**, $t = 2.926$, $p<0.01$). VPA treatment had no significant effect on anxiety-related behavior in wild type mice. **D-E)** Depression related behavior following VPA administration was assessed in both the forced swim test (FST,**D**) and learned helplessness (LH,**E**). Analysis by two-way ANOVA revealed that *Clock*Δ19 mice spend less time immobile on the FST (Main effect of genotype; $F_{(1,55)} = 8.03$, $p = 0.0064$) and failed to escape fewer times in the LH (Main effect of genotype; $F_{(1,49)} = 4.15$, $p = 0.047$). Bonferroni post test revealed that VPA treatment caused a significant increase in immobile time on the FST (**D**, $t = 2.823$, $p<0.05$) and in failures to escape in the LH (**E**, $t = 2.683$, $p<0.05$);(n = 12-15 per group).

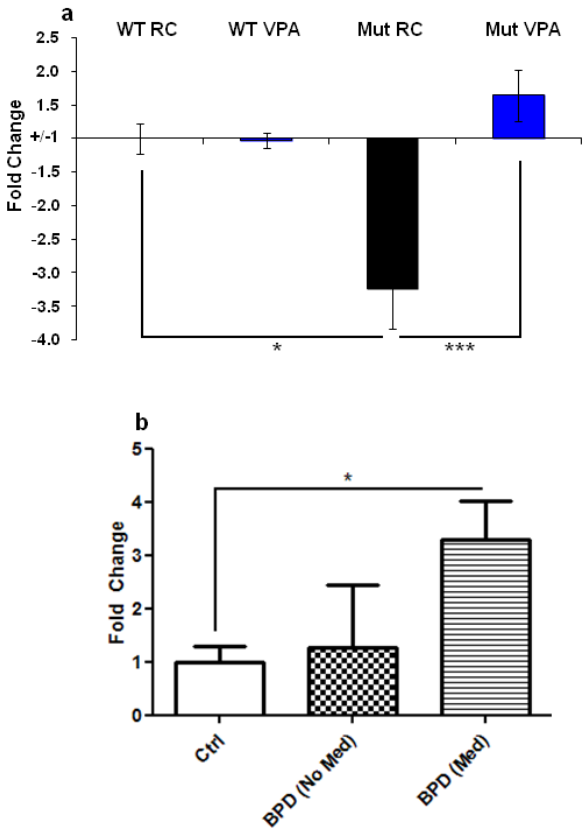


Figure 3-2. Regulation of *Cck* levels in the VTA by mood stabilizers. A) Relative mRNA levels of *Cck* in *Clock*Δ*19* (Mut) mice and WT littermates receiving 2 weeks of VPA or RC. Levels were normalized to an internal control, *Gapdh*. Analysis by two-way ANOVA revealed a significant decrease in *Cck* mRNA levels in untreated *Clock*Δ19 mice compared to WT animals (main effect of genotype $F_{1,29}$ =4.59; p=0.0406). Bonferroni post hoc tests revealed that VPA treatment caused a significant increase in *Cck* expression in *Clock*Δ19animals, restoring it to near WT levels (t=5.065, p<0.001). VPA treatment had no detectable effect of WT *Cck* expression; (n = 7-10 per group). **B)** Relative mRNA levels of *Cck* in the VTA of BPD patients,either receiving (Med) or not receiving medication (No Med), and normal controls. Levels were normalized to an internal control, *Gapdh*. Analysis by one-way ANOVA a significant difference in means (F=4.5254, p = 0.0266). Bonferroni post hoc tests revealed that BPD patients receiving medication had significantly higher levels of *Cck* mRNA in the VTA than control patients (t = 2.903, p<0.05). *Cck* levels in BPD patients not receiving medication did not differ significantly from either groups; (n = 9 for control, n= 3 for BPD (No Med), n = 8 for BPD (Med)).

Patient	Sex	Age	Race	DX	PMI	RIN
002-09	M	46	C	NC	22	9.6
002-08	M	47	C	NC	24	8.2
030-07	M	52	C	NC	15	6.3
012-08	M	51	C	NC	19	7.8
016-08	M	52	C	NC	23	8.3
021-08	M	50	C	NC	16	6.5
027-08	M	41	C	NC	24	9.3
028-08	M	46	C	NC	21	8.3
026-07	M	52	C	NC	24	6.1
002-06	M	28	C	BPD	22	9.4
004-06	M	40	C	BPD	8	7
001-08	F	54	C	BPD	17	6.2
023-08	M	27	C	BPD	23	7.3
004-09	M	36	C	BPD	11	8.9
023-09	M	30	C	BPD	22	8.5
08-010	M	47	C	BPD	17	8.6
003-10	M	34	B	BPD	12	5
008-10	M	33	C	BPD	22	6.5
009-10	M	33	C	BPD	16	7.7
011-10	F	50	C	BPD	29	5.3

Table 3-1. Individual human postmortem tissue subjects. Data for each of the individual patients from which the postmortem VTA samples were obtained.

FACTOR	CONTROLS	BPD	STUDENTS T-TEST
AGE	46.5 ± 7.43	37.5 ± 9.15	0.022
PMI	21 ± 3.30	18.1 ± 6.20	0.19
RIN	7.98 ± 1.30	7.31 ± 1.47	0.28

Table 3-2. Averaged data for patient groups. Averaged data for BPD and control groups showing no significant differences between BPD and control groups in postmortem interval (PMI), or RNA Integrity Number (RIN). BPD groups and control groups differed significantly in age; however, Pearson product-moment correlation coefficient to were calculated determine if there were differences in Cck mRNA with age. There was no significant correlation with *Cck* mRNA levels and age in either Control (Pearsons r = -0.2186; p= 0.5721) or BPD (Pearsons r = 0.01669; p= 0.9635) groups.

PATIENT	SEX	AGE	RACE	DX	MEDICATION
001-08	F	54	C	BPD	None
023-08	M	27	C	BPD	Seroquel, Li
004-09	M	36	C	BPD	None
023-09	M	30	C	BPD	Doxepin
08-010	M	47	C	BPD	Celexa, Prolixin
003-10	M	34	B	BPD	WBT, Zoloft
008-10	M	33	C	BPD	Celexa, Depakote
009-10	M	33	C	BPD	None
011-10	F	50	C	BPD	Geodon

Table 3-3. Medication status of BPD human postmortem tissue subjects. BPD patients were further separated into those on or off medication. Medications included antidepressants (Doxepin, Celexa, WBT, Zoloft), mood stabilizers (Seroquel, Geodon, Li, Depakote), and antipsychotics (Prolixin).

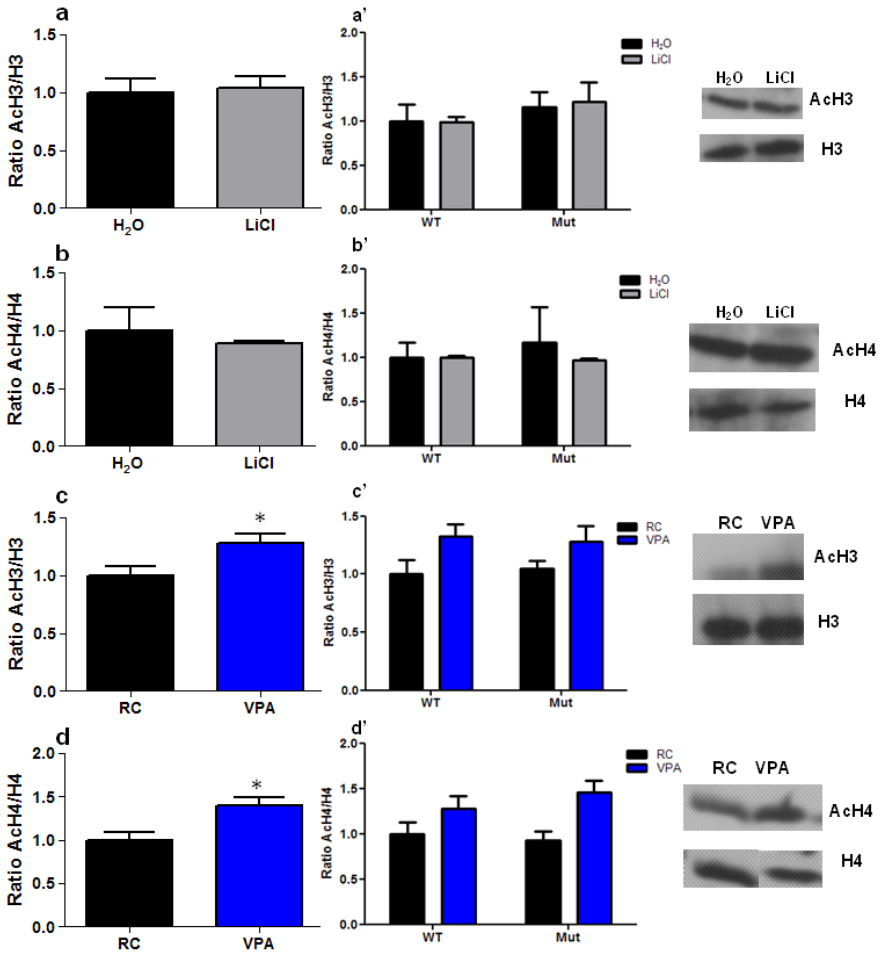


Figure 3-3. Regulation of levels of histone acetylation in the brain of *Clock*Δ19 mice by mood stabilizers. Western blots were performed on extracted histones from the VTA-containing midbrain sections from *Clock*Δ19 (Mut) mice and WT controls receiving lithium (LiCl) (**A,B**) or VPA (**C,D**) treatment and their respective controls and levels of acetylated histone H3 (**A,C**)and H4 (**B,D**) were assessed. Lithium treatment had no detectable effects on levels of AcH3 (**A,** t_{22}=0.2417, p = 0.8113) or AcH4 (**B**, t_9 = 0.3863, p = 0.7083) when groups were divided into treated (LiCl) or untreated (H_2O) (n = 10-12 per group) or when separated by genotype and treatment (**a'**$F_{1,20}$ = 0.03, p = 0.8347; **b'**$F_{1,7}$ = 0.09, p = 0.7674); (n = 4-6 per group). Representative bands are shown to the right. **C)** VPA treated animals had a significant increase in levels of AcH3 (t_{18} = 2.373, p = 0.0290; (n = 9-11per group). This increase was present in both genotypes (**c'**) and analysis by two-way ANOVA revealed a main effect of treatment ($F_{1,16}$= 4.51, p = 0.0496; (n=4-6 per group)) on levels of AcH3. Representative Bands are shown to the right. **D)** VPA treated animals had a significant increase in levels of AcH4 (t_{19} = 2.816, p = 0.0110; (n = 9-12per group). This increase was present in both genotypes (**d'**) and analysis by two-way ANOVA revealed a main effect of treatment ($F_{1,17}$= 7.48, p = 0.0141;(n=4-6 per group)) on levels of AcH4. Representative bands are shown to the right.

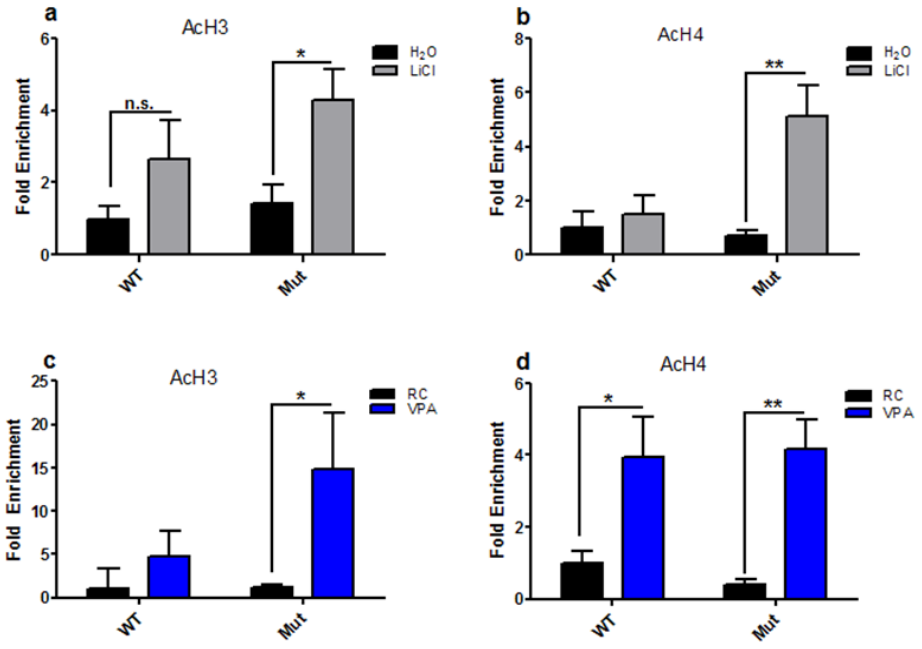


Figure 3-4. Mood stabilizers regulate levels of histone acetylation at the *Cck* promoter. Relative levels of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) at the *Cck* promoter in *Clock*Δ*19* (Mut) mice and WT littermates following lithium (LiCl,**A,B**) or VPA (**C,D**) treatment were assessed by performing ChIP assays with a AcH3 and AcH4 specific antibodies. **A-B)** Analysis by two-way ANOVA revealed a main effect of lithium treatment on AcH3(**A**,$F_{1,20}$=9.4, p=0.0061) and AcH4(**B**,$F_{1,17}$=11.32, p=0.0037) levels. Bonferroni post-tests revealed a significant increase in levels of AcH3 (t = 2.744, p<0.05) and AcH4 (t=4.198, p<0.01) at the Cck promoter in *Clock*Δ*19* mice following lithium treatment, while lithium had no detectable effect on WT animals; (n= 5-6 per group). **C-D)** Analysis by two-way ANOVA revealed a main effect of VPA treatment on AcH3(**A**,$F_{1,16}$=5.27, p=0.0356) and AcH4(**B**,$F_{1,20}$=22.68, p=0.0001) levels. Bonferroni post-tests revealed a significant increase in levels of AcH3 (t = 2.690, p<0.05) and AcH4 (t=3.781, p<0.01) at the Cck promoter in *Clock*Δ*19* mice following VPA treatment. VPA also caused a significant increase in levels of AcH4 (t=2.954, p<0.05) and a non-significant increase in AcH3 (t = 0.661, p>0.05) at the Cck promoter; (n= 5-6 per group).

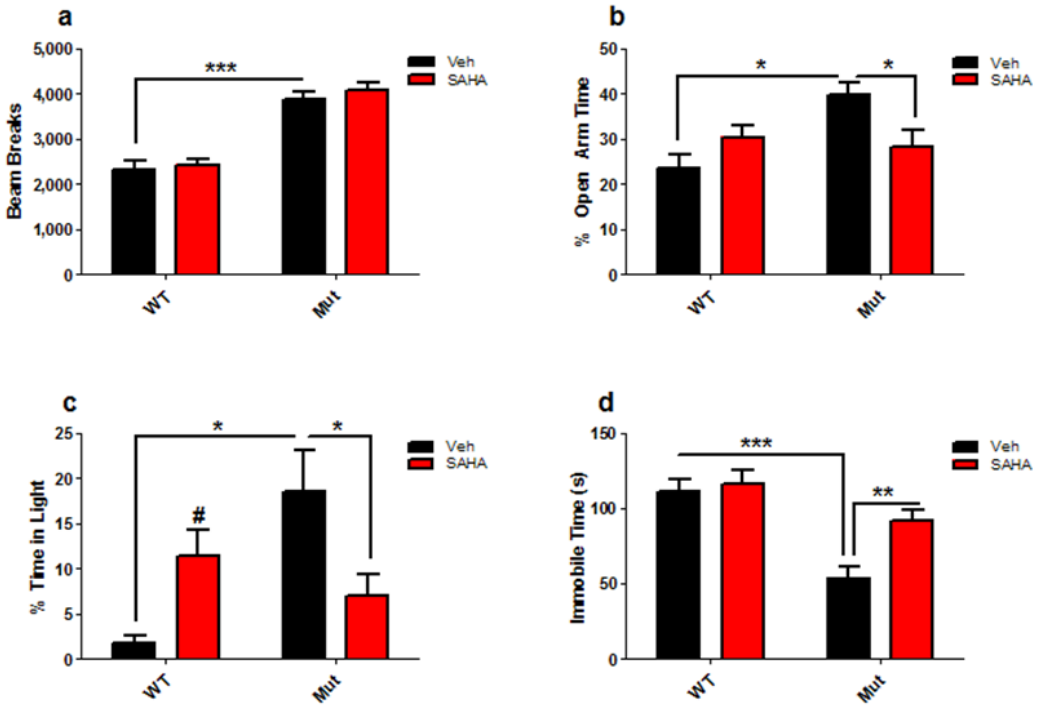


Figure 3-5. Administration of the specific HDACi SAHA mimics the therapeutic effects of VPA on *Clock*Δ19 behavior. A) Locomotor activity was measured in *Clock*Δ19 (Mut) mice and wild type (WT) littermates for two hours following 2 weeks of SAHA or HOP- β-CD vehicle (Veh). Analysis by two-way ANOVA revealed a significant main effect of genotype ($F_{(1,76)}$ = 82.40, p< 0.0001). Bonferroni post-tests revealed that there was no significant effect of any treatment on locomotor response to novelty. **B-C)** Anxiety related behavior following SAHA administration was assessed in both the elevated plus maze (EPM,**B**) and dark/light test (**C**). Analysis by two-way ANOVA revealed that *Clock*Δ19 mice spend more time in the open arms of the EPM (Main effect of genotype; $F_{(1,68)}$ = 5.55, p = 0.0214) and light side of the dark/light box (Main effect of genotype; $F_{(1,67)}$ = 4.85, p = 0.0311). Bonferroni post tests revealed that SAHA treatment caused a significant reduction in open arm time in the EPM (**B,** t = 2.682, p<0.05) and in time spent in the light side of the dark/light test (**C**, t = 2.764, p<0.05). In WT mice, SAHA treatment had no significant effect on time spent in the open arm of the EPM, but caused a significant increase in time spend in the light side of the dark/light box(**C**, t = 2.605, p<0.05). **D)** Depression related behavior following SAHA administration was assessed in the forced swim test (FST). Analysis by two-way ANOVA revealed that *Clock*Δ19 mice spend less time immobile on the FST (Main effect of genotype; $F_{(1,63)}$ = 25.59, p<0.0001). Bonferroni post test revealed that SAHA treatment caused a significant increase in immobile time on the FST (t = 3.329, p<0.01); (n = 15-20 per group).

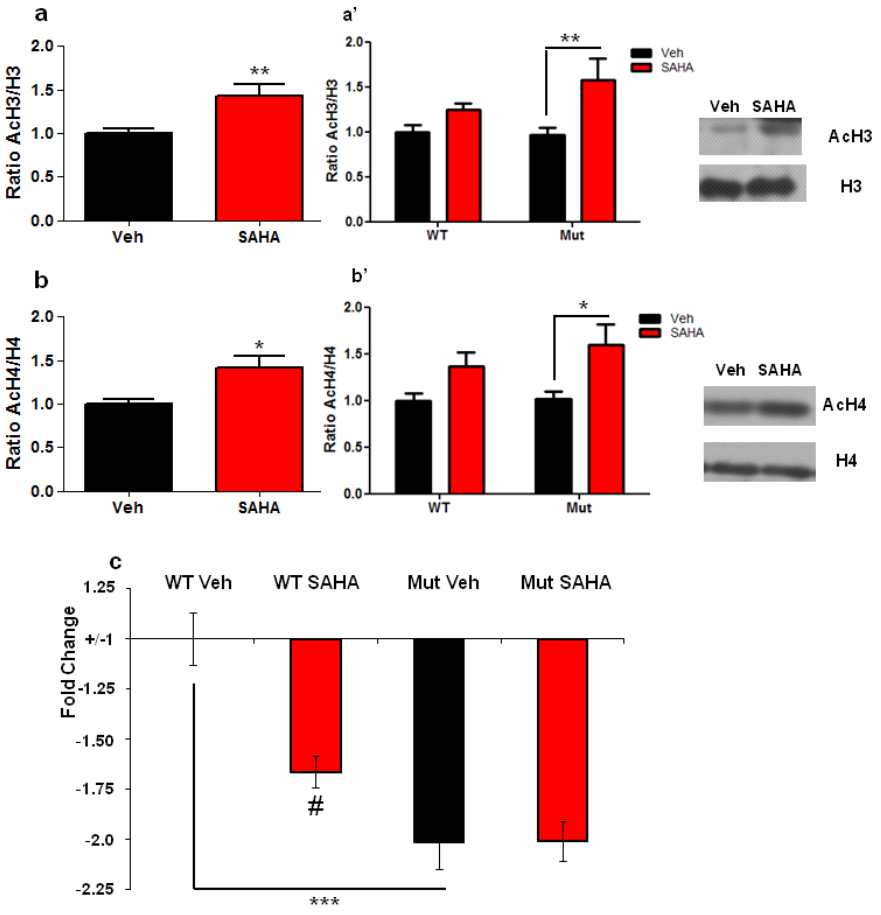


Figure 3-6. Molecular effects of SAHA administration in the *Clock*Δ19 mice. A,B)Western blots were performed on extracted histones from the VTA-containing midbrain sections from *Clock*Δ19 mice and WT controls receiving SAHA or HOP-β-CD vehicle (Veh). **A)** SAHA treated animals had a significant increase in levels of AcH3 (t_{23} = 3.169, p = 0.0043; (n = 12-13per group). This increase was present in both genotypes (**a'**) and analysis by two-way ANOVA revealed a main effect of treatment ($F_{1,21}$= 10.59, p = 0.038; (n=6-7 per group)) on levels of AcH3. Bonferroni post-tests revealed a significant increase in levels of AcH3 in *Clock*Δ19 mice (t = 3.232, p<0.01) . Representative Bands are shown to the right. **B)** SAHA treated animals had a significant increase in levels of AcH4 (t_{19} = 2.848, p = 0.0103; (n = 10-11 per group). This increase was present in both genotypes (**b'**) and analysis by two-way ANOVA revealed a main effect of treatment ($F_{1,17}$= 11.62, p = 0.0033;(n=5-6 per group)) on levels of AcH4. Bonferroni post-tests revealed a significant increase in levels of AcH3 in *Clock*Δ19 mice (t = 3.034, p<0.05) Representative bands are shown to the right. **C)** Relative mRNA levels of *Cck* in *Clock*Δ*19* mice and WT littermates receiving 2 weeks of SAHA or HOP-β-CD vehicle (Veh). Levels were normalized to an internal control, *Gapdh*. Analysis by two-way ANOVA revealed a significant decrease in *Cck* mRNA levels in untreated *Clock*Δ19 mice compared to WT animals (main effect of genotype $F_{1,20}$ = 27.30; p<0.0001). Bonferroni post hoc tests revealed that SAHA treatment has no detectable effect on Cck levels in *Clock*Δ19 mice, but caused a significant decrease in WT Cck mRNA levels (#, t=4.889, p<0.001); (n = 5-6 per group)

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Bipolar disorder (BPD) is a severe and chronic psychiatric disease. Due to its complex nature, the mechanisms that contribute to the development of this disease, and the actions by which mood stabilizers exert therapeutic effects in the treatment of BPD, remain poorly understood. Recent genetically manipulated mouse models of BPD, in particular the *Clock*Δ19 mice, have allowed for an opportunity to better study this disease. Because the *Clock*Δ19 mice have a behavioral phenotype that is strikingly similar to human mania, and respond to the mood stabilizer lithium, they can be used to determine how manic-like behaviors develop as well as more accurately elucidate the mechanisms of action of mood stabilizers, because they model a pre-existing disease state, similar to BPD. My thesis work was concerned with identifying molecules that might be involved in the development of the manic-like behaviors of the *Clock*Δ*19* mice, as well as elucidating mechanisms by which these molecules are regulated. In addition, we also focused on determining molecules that are involved in the treatment of these manic-like behaviors, and identified potential mechanisms of action of mood stabilizers.

Because CLOCK is a transcription factor, we set out to identify potential CLOCK target genes, based on altered transcription in the *Clock*Δ19 mice compared to WT animals. My work focused primarily on the peptide neurotransmitter *Cck*, which is decreased in the VTA of *Clock*Δ19 mice, and has been linked to several of the behaviors that are disrupted in the *Clock*Δ19 mice. We determined that *Cck* is indeed a CLOCK target gene, and that CLOCK normally functions as a positive regulator of the *Cck* gene.

Therefore, the decrease in *Cck* expression observed in the VTA of *Clock*Δ19 mice is due to the dominant negative function of the CLOCKΔ19 protein. More specifically, this decrease is due to the inability of CLOCKΔ19 to interact with the histone methyltransferase (HMT) MLL1, which was found to be decreased at the *Cck* promoter in the *Clock*Δ19 mice. A decrease in MLL1 levels results in a reduction in the histone modification H3K4me3 at the *Cck* promoter, which has been found to be highly linked to transcriptional activation (Martin and Zhang 2005; Katada and Sassone-Corsi 2010). This regulation of the *Cck* gene in the *Clock*Δ19 mice is modeled in Figure 4-1.

The loss of CLOCK regulation at the *Cck* promoter and subsequent decrease in *Cck* gene expression in the VTA is particularly important in the development of the *Clock*Δ19 phenotype. AAV-mediated knockdown of *Cck* specifically in the VTA of WT C57/Bl6 mice results in a manic-like behavioral profile that is similar to the *Clock*Δ19 mice, indicating that a decrease in *Cck* in the VTA is sufficient to induce manic-like behaviors. These findings are in accord with what has been previously shown by others examining the effects of systemic administration and intracerebralventricular administration of CCK, as well as CCK_B receptor agonists and antagonists (Rotzinger, Lovejoy et al. 2010); however, we have localized the ability of CCK to these behaviors to a single brain region, the VTA.

We have characterized the effects of *Cck* knockdown on mood-related behavior, but CCK is also known to be involved in reward as well. Administration of CCK_B receptor agonists decrease the reward magnitude of self-administered psychostimulants (Rotzinger, Bush et al. 2002). It will be interesting in the future to examine whether decreased *Cck* levels in the VTA also play a role in the hyperhedonic phenotype observed

in the *Clock*Δ19 mice. This can be examined by using the AAV-Cck-shRNA created in the current work to knock-down *Cck* in the VTA of WT animals, and these animals can be subjected to behavioral tests to assess their hedonic state, including the sucrose preference test and conditioned place preference for drugs such as cocaine. An increase in preference for rewarding stimuli in mice infected with AAV-Cck-shRNA would indicate that a decrease in *Cck* is likely involved in the reward phenotype of the *Clock*Δ19 mice.

Additionally, *Cck* is known to inhibit dopaminergic transmission (Voigt and Wang 1984). The *Clock*Δ19 mice have an increase in VTA dopamine (DA) cell firing, which may be important for the development of their phenotype. AAV-mediated knockdown of CLOCK in the VTA of WT mice results in a recapitulation of several manic-like behaviors (Mukherjee, Coque et al. 2010). Additionally, cells infected with AAV-Clock-shRNA have an increased DA cell firing rate (Mukherjee, Coque et al. 2010). Unpublished studies in our lab using optogenetics find that a chronic increase in the firing rate in the VTA of WT animals also recapitulates some of the *Clock*Δ19 behaviors. In the future, it will be interesting to see the effects of *Cck* knockdown on DA cell firing, to see if it causes a similar increase, suggesting this may be a way that it is causing some of these manic-like behaviors.

Though we have identified *Cck* as a CLOCK target that is important for the development of manic-like behaviors in the *Clock*Δ19 mice, other molecules are also involved in their phenotype. In the future it will be interesting to identify other CLOCK targets that may be important for the development of manic-like behaviors. To identify these targets, we can perform Chromatin immunoprecipitation-sequencing (ChIP seq). In

this technique, DNA sequences enriched by a transcription factor are isolated by ChIP and then subjected to high throughput sequencing (Aleksic and Russell 2009) . Utilizing this technique in both WT and *Clock*Δ19 mice, we can identify a multitude of CLOCK target genes in both genotypes. This ChIP-seq analysis can then be compared to RNA-seq from WT and *Clock*Δ19 mice. In RNA-seq, RNA is subjected to high throughput sequencing, allowing for whole transcriptome profiling. By comparing ChIP-seq and RNA-seq, it can then be determined if genes that are differentially expressed in *Clock*Δ19 mice and WT mice are CLOCK targets. We can then attempt to mimic those changes observed in the *Clock*Δ19 mice, utilizing viral mediated gene transfer to either knockdown or overexpress a gene, in WT animals and assess whether manic-like behaviors develop.

We have also determined that lithium causes a selective increase in *Cck* levels, and that this increase is necessary for lithium's therapeutic actions as *Clock*Δ19 mice injected with AAV-Cck-shRNA into the VTA no longer respond to lithium treatment. It will be interesting to further examine the role of *Cck* and more specifically CCK receptor subtypes in the treatment of manic-like behaviors. Future studies could examine the effects of chronic administration of both CCK_A and CCK_B receptor agonists to the *Clock*Δ19 mice, to determine if stimulation of a specific subtype of CCK receptor alone also has an "anti-manic" effect similar to lithium. If CCK receptor agonists have therapeutic effects in the *Clock*Δ19 mice, it would also be interesting to determine which specific projections from the VTA regulate distinct mood stabilizing actions of lithium. To determine this, osmotic minipumps could be implanted into different brain regions of *Clock*Δ19 mice to allow for a chronic infusion of CCK receptor agonists and behavioral

testing could be carried out. Brain regions of interest include the amygdala, nucleus accumbens, and cortex, as CCK receptors have been found to regulate anxiety, locomotor activity, and depression related behaviors in these regions (Rotzinger and Vaccarino 2003; Becker, Zeau et al. 2008; Kim, Lee et al. 2008; Rotzinger, Lovejoy et al. 2010).

In addition to identifying the importance of the *Cck* gene in lithium's therapeutic actions, we have also begun to determine the mechanism by which lithium causes a selective increase in *Cck* mRNA levels in the *Clock*Δ19 mice. This mechanism involves displacement of the CLOCKΔ19 protein from the *Cck* promoter and recruitment of chromatin remodeling enzymes (Figure 4-1). More specifically, we have found that the decreased levels of H3K4me3 at the *Cck* promoter are restored to WT levels in *Clock*Δ19 mice and that there is a selective increase in levels of acetylated histones H3 and H4 (AcH3, AcH4) following lithium treatment (Figure 4-1). The increase in H3K4me3 may be partially due to MLL1, as there is a trend towards an increase at MLL1 levels at the *Cck* promoter in lithium treated *Clock*Δ19 mice; however other histone methyltransferases are likely involved. The increase in AcH3 and AcH4 is likely due to the recruitment of histone acetyltransferases (HATs), which may be recruited by the factors that displace CLOCKΔ19 from the *Cck* promoter following lithium treatment. One potential hypothesis for the selective increase in both of these chromatin modifications involves crosstalk between H3K4me3 and histone acetylation. In *Clock*Δ19 mice following lithium treatment a transcriptional factor complex, that may be associated with either a HAT or HMT, displaces the CLOCKΔ19 protein, causing increases in histone acetylation or methylation. Increases in either modification can cause concomitant increases in the other. For example, MLL-family member MLL4

recognizes AcH3/4 and deposits H3K4me3 at promoters where this is found (Nightingale, Gendreizig et al. 2007). Conversely, the HATs CBP and p300 are known to be recruited to promoters that contain H3K4me3 (Crump, Hazzalin et al. 2011). In future studies, ChIP assays can be performed to assess if there are any possible changes in these proteins, and other HATs or HMTs, at the *Cck* promoter following lithium treatment.

In order to better understand what specific chromatin remodeling complexes are being recruited to the *Cck* promoter in lithium treated *Clock*Δ19 mice, it will be important to identify the transcription factor or factors that are regulated by lithium in the *Clock*Δ19 mice, as they will help elucidate the precise mechanism by which lithium is regulating the *Cck* gene. Though we have established that there is a decrease in CLOCKΔ19 at the *Cck* promoter following lithium treatment, it is necessary to determine if lithium also causes a decrease in BMAL1 levels at the *Cck* promoter using ChIP assays. If BMAL1 levels at the *Cck* promoter are unchanged, then transcriptional activation of the *Cck* gene via E-box binding proteins may be important for lithium's regulation of *Cck*. It is possible that lithium treatment may increase levels or activity of other BMAL1 binding partners, such as the CLOCK analogue NPAS2, and the transcription factors Endothelial PAS domain protein 1 (EPAS1) and Hypoxia inducible factor 1, alpha subunit (HIF1α), which can compete with CLOCKΔ19 (Hogenesch, Gu et al. 1998).

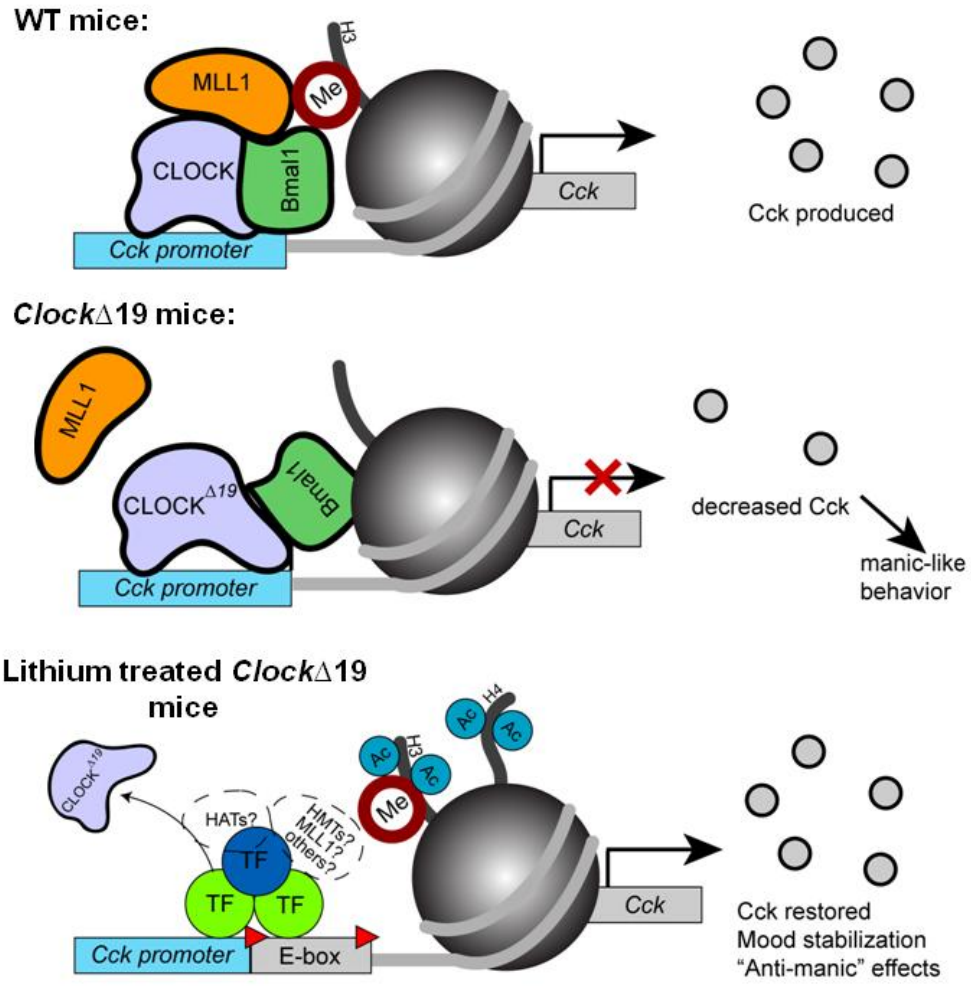


Figure 4-1. Hypothetical Model of Regulation of the *Cck* Gene in *Clock*Δ19 mice. In WT animals, CLOCK/BMAL1 bind to the *Cck* promoter, recruiting MLL1 which deposits H3K4me3, allowing for activation of the *Cck* gene and normal levels of *Cck* to be produced. In *Clock*Δ19 mice, the mutant CLOCKΔ19/BMAL1 are still bound to the *Cck* promoter, but can no longer interact with MLL1, resulting in a decrease in H3K4me3 at the *Cck* promoter and decreased transcription of the *Cck* gene. This decrease in *Cck* levels contributes to the manic-like behavior of the *Clock*Δ19 mice. When *Clock*Δ19 mice receive lithium treatment, CLOCKΔ19 is displaced from the *Cck* promoter and an unknown transcription factor complex is recruited to the *Cck* promoter. Lithium treatment also causes an increase in levels of H3K4me3 and levels of acetylated histone H3 and H4, potentially through the recruitment of MLL1 and other histone methyltransferases (HMTs) and histone acetyltransferases (HATs) by this transcription factor complex. Following the recruitment of these factors, *Cck* gene transcription is increased and *Cck* levels are restored to near WT, resulting in the "anti-manic" effects of lithium in the *Clock*Δ19 mice.

In addition to the E-box, the nearby CRE site is an enticing candidate, as luciferase assays have found the CRE site to be important for basal activity of a *Cck* luciferase reporter (Enwright, Wald et al. 2010); however, this effect is not mediated by the transcription factor CREB, as we have found that using ChIP assays that *Cck* is not a CREB target gene in the VTA or other brain regions (Figure 4-2;(Enwright, Wald et al. 2010)) .

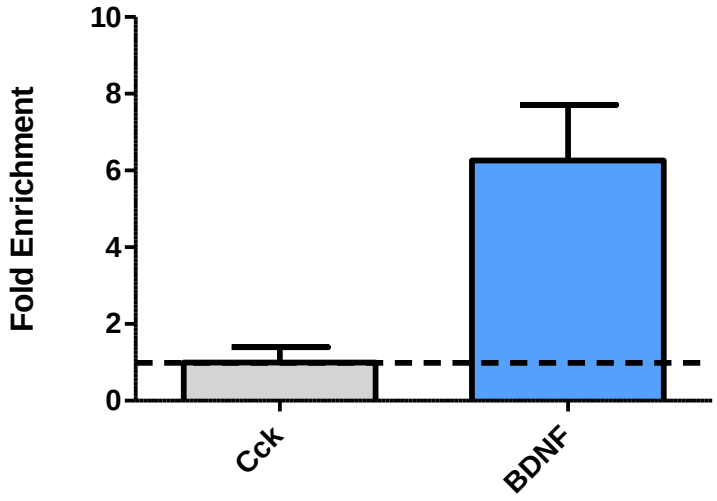


Figure 4-2. CREB does not bind to the *Cck* promoter. Fold enrichment at proximal promoter region following ChIP with a CREB specific antibody comparing *Clock*Δ*19* mutants and wild-type (WT) littermate controls. One sample t-tests revealed that CREB is not significantly enriched at the *Cck* promoter region above background. As a positive control fold enrichment was also calculated for a known CREB target gene, BDNF. As expected, there was a significant enrichment of CREB at the BDNF promoter (~6 fold) above background (t_4=3.621, p = 0.0223).

In the future it will be interesting to determine if lithium is affecting *Cck*, and other targets, via pathways that lithium has been previously hypothesized to regulate. This can be accomplished by mimicking lithium's hypothesized effects on a particular signaling pathway in the *Clock*Δ19 mice, and determine if behavioral rescue similar to lithium can be achieved. We have begun to investigate the effects of chronic administration of a

specific GSK-3β inhibitor to *Clock*Δ19 mice. Interestingly, this compound acts to decrease *Clock*Δ19 locomotor activity (Kozikowski, Gunosewoyo et al. 2011). It also has no detectable effects on *Clock*Δ19 anxiety or depression related behavior, though there is a trend towards this compound producing antidepressant-like effects in the forced swim test (unpublished observations). These effects are similar to what is observed when GSK-3β function is reduced in WT animals, either by pharmacological inhibition or genetic haploinsufficiency, but do not mimic the behavioral effects of lithium treatment in the *Clock*Δ19 mice, suggesting that GSK-3β inhibition may not play a role in lithium's mood stabilizing actions in these mice (Gould, Einat et al. 2004; Kaidanovich-Beilin, Milman et al. 2004; O'Brien, Harper et al. 2004; Roybal, Theobold et al. 2007).

Another pathway of interest is for future studies is inositol signaling. Lithium inhibits inositol monophosphatase (IMPase), so the effects of chronic administration of specific IMPase inhibitors, such as L-690,330, on *Clock*Δ19 behavior could be determined (Atack, Cook et al. 1993). An alternative to this would be the administration of antagonists of inositol 1,4,5-tris-phosphate (IP_3) receptors like xestospongin C, which would mimic the effects of inositol depletion by lithium, which causes a reduction in IP_3 levels (Galeotti, Vivoli et al. 2008). Unfortunately, these compounds do not readily cross blood brain barrier, unlike lithium, and may be difficult to study via systemic administration (Atack, Cook et al. 1993; Galeotti, Vivoli et al. 2008). An alternative approach to systemic administration would be intracerebroventricular (i.c.v.) administration of these compounds, which have the advantage of limiting any observed effects on behavior to inhibition of inositol signaling solely in the brain.

In addition to identifying molecular actions of lithium, we have determined that the mood stabilizer valproate (VPA) also has therapeutic actions in the *Clock*Δ19 mice, further validating them as a predictive model of mania. Because lithium and VPA had similar behavioral effects, we hypothesized that they may have similar molecular targets, such as *Cck*, which was found to be important for lithium's "anti-manic" effects. Similar to lithium treatment, we found that VPA treatment regulates the *Cck* gene,causing a selective increase in *Cck* mRNA in the *Clock*Δ19 mice while having no detectable effect on WT animals. Though this suggests that *Cck* may be a gene that is important for the actions of VPA as well, in the future it will need to be determined if an increase in *Cck* in the VTA is also necessary for VPA's actions. Gene knockdown studies similar to the ones performed in Chapter 2 can be performed to determine if the knockdown of *Cck* in the VTA of *Clock*Δ19 mice prevents the therapeutic actions of VPA.

This increase in *Cck* levels in the VTA is common to mood stabilizers in the *Clock*Δ19 mice, and based upon studies performed on VTA tissue from human BPD patients, it may be a common target for current BPD treatments as well. There was a consistent increase in *Cck* mRNA in the VTA of BPD patients receiving mood stabilizers, antidepressants, and antipsychotics. We have also begun investigating whether this increase in *Cck* is specific to the VTA in human postmortem tissue or if other brain regions could be affected. Preliminary studies have been performed to assess *Cck* levels in the nucleus accumbens (NAc) tissue from human BPD patients (Figure 4-3). We chose to investigate the NAc because it is part of the mesolimbic dopamine circuit that may be disrupted in BPD. Interestingly, *Cck* levels appear to be decreased in the NAc of BPD patients not receiving medications when compared to normal control subjects, while

in BPD patients receiving medications, *Cck* mRNA levels were increased to near those of normal control subjects (Figure 4-3). This is similar to what is observed in the *Clock*Δ19 mice, Cck mRNA levels in the NAc of more subjects need to be assessed to confirm these findings. Future studies will determine if an increase in *Cck* is specific to BPD patients receiving medications or occurs following treatment in psychiatric diseases that share some similar characteristics and pharmacological treatments with BPD, like major depressive disorder or schizophrenia.

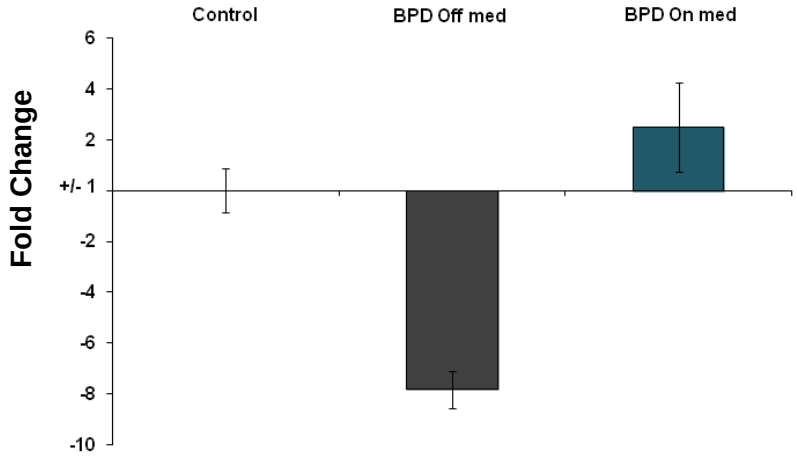


Figure 4-3. Assessment of Cck Levels in the Nucleus Accumbens of Human BPD Subjects
Relative mRNA levels of *Cck* in the VTA of BPD patients,either receiving (Med) or not receiving medication (No Med), and normal controls. Levels were normalized to an internal control, *Gapdh*. *Cck* levels appear to be decreased in the NAc of BPD patients not receiving medications when compared to normal control subjects, while in BPD patients receiving medications *Cck* mRNA levels were increased to near those of normal control subjects; n= 3 per group (Controls and BPD On Med) or 2 per group (BPD Off Med).

We also found that VPA may be regulating target genes through HDAC inhibition, and VPA administration causes an increase in histone acetylation throughout the brain, as well as at the *Cck* promoter, in both WT and *Clock*Δ19 animals. However, it still remains possible that VPA can affect other targets than HDACs, and can also regulate gene transcription in another manner. However, we were able to establish that HDAC inhibition can be responsible for VPA's mood stabilizing effects because administration of the specific Class I and IIb HDACi SAHA caused a behavioral rescue in the *Clock*Δ19 similar to VPA. This is in accord with the previous findings that the non-specific HDACi sodium butyrate (SB) can prevent and reverse amphetamine induced hyperactivity in WT rats (Moretti, Valvassori et al. 2011); however, our results confirm that mood stabilizing effects can be due exclusively to inhibiting HDACs in a genetic model of mania.

However, SAHA administration did not fully mimic the molecular effects of VPA treatment in *Clock*Δ19 animals, because *Cck* levels were unaffected in SAHA treated *Clock*Δ19 mice. These results suggest that inhibition of Class I and IIb HDACs, more specifically the SAHA targets HDAC1,2,3,and 6 (Bradner, West et al. 2010), is sufficient to rescue *Clock*Δ19 manic-like behavior, but it is possible that VPA may also regulate behavior of the *Clock*Δ19 mice through inhibition of ClassIIa HDACs (Figure 4-4). Future studies will focus on identifying specific HDACs that are most important for the mood stabilizing actions of VPA and SAHA. To do this other HDAC inhibitors can be administered to the *Clock*Δ19 mice to determine if they can affect manic-like behaviors. These can include: TSA (Class I: HDAC1,3; IIa: HDAC4; IIb:HDAC6,10), MS-275 (Class I:HDAC1 over HDAC3), RGFP136 (Class I: HDAC3), MC1568 (Class

II, HDAC4,5), NQN-1 (ClassIIb, HDAC6), and pandacostat, a recently developed pan-HDAC inhibitor (Bradner, West et al. 2010). Alternatively, individual HDACs or combinations of HDACs could be knocked down in the brain of *Clock*Δ19 mice by i.c.v. administration of antisense oligonucleotides directed against specific HDACs.

Based on the results of the present study, we now are aware of three compounds that can rescue manic-like behaviors of *Clock*Δ19 mice: lithium, VPA, and SAHA. Future studies can attempt to identify common targets of these treatments. Gene expression profiling by RNA-seq can be performed on *Clock*Δ19 mice receiving lithium, VPA, or SAHA and the results can be compared to see what is commonly and differentially regulated by these compounds. A similar gene expression analysis can be performed in WT animals receiving lithium, VPA, and SAHA to identify molecules that are regulated selectively by treatment in the *Clock*Δ19 mice, as they may be the most attractive candidate mood stabilizer targets.

In the case of VPA and SAHA, once potential important target genes have been identified, it can be determined if the genes are regulated by HDAC inhibition by performing ChIP assays measuring levels of acetylated histones at these promoters. Additionally, if specific HDACs have been identified that are important for the actions of VPA and SAHA, then it can be determined by ChIP assays which VPA and SAHA target genes are regulate by certain HDACs. Once more is learned about the transcription factors and chromatin remodeling enzymes that are regulated by lithium treatment, similar studies can be carried out.

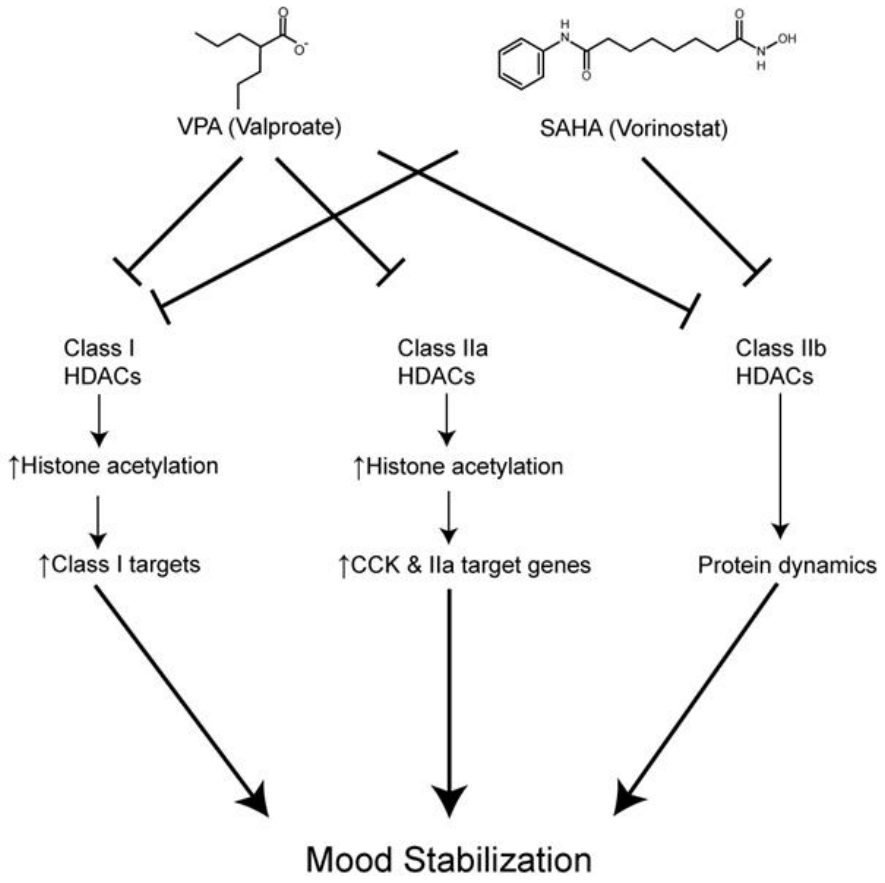


Figure 4-4. Schematic diagram of potential mechanisms for the mood stabilizing effects of HDAC inhibitors in *Clock*Δ19 mice. VPA administration inhibits Class I and II HDACs, which increases levels of histone acetylation of ClassI target genes, and Class IIa target genes, which may include *Cck*, resulting in increases in transcription of these genes and mood stabilizing effects. SAHA administration results in similar molecular effects, increasing levels of histone acetylation of ClassI target genes and gene transcription. VPA and SAHA may also have mood stabilizing effect by inhibition of Class IIb HDACs, which can affect protein dynamics, such as microtubule formation, protein folding and turnover.

Interestingly both lithium and VPA can affect circadian rhythms, by lengthening the circadian period (Klemfuss 1992; Dokucu, Yu et al. 2005). It is unclear whether or not this is important for mood stabilizing actions, but regulating the rhythms of BPD patients in psychosocial therapies like Interpersonal and Social Rhythm Therapy (IPSRT)

leads to improved patient outcomes in a shorter amount of time than traditional psychotherapy (Frank, Swartz et al. 2000). We have also recently found that administration of a Casein Kinase1 epsilon/delta (CK1ε/δ) inhibitor, CK01, partially rescues the manic-like behaviors of *Clock*Δ19 mice (Appendix A). CK1ε/δ inhibitors produce a phase delay and period lengthening in animals (Badura, Swanson et al. 2007; Meng, Maywood et al. 2010; Sprouse, Reynolds et al. 2010), which is similar to the effects of lithium and VPA which can lengthen the circadian period. CK1ε/δ inhibitors can also stabilize rhythms in arrhythmic mice (Meng, Maywood et al. 2010). It would be interesting to see if regulation of circadian rhythms plays a role in the mood stabilizing actions of lithium, VPA, and CK01. Circadian rhythms of *Clock*Δ19 and WT mice during administration of the drugs can be assessed to determine if the arrhythmic phenotype of the *Clock*Δ19 mice is ameliorated by treatment. It would also be interesting to determine if there are any potential changes in molecular rhythms following lithium, VPA, or CK01 treatment. Gene expression analysis can be performed at several timepoints across the day to determine if previously observed changes in mood stabilizer target genes are due to altering rhythms or overall levels of gene expression.

In conclusion, we have identified a novel CLOCK target, *cholecystokinin*, that is important for the development and treatment of manic-like behaviors in the *Clock*Δ19 mice. Future studies will attempt to identify other CLOCK target genes that may also be involved in the *Clock*Δ19 phenotype. In addition, we have identified a role for the regulation of chromatin remodeling in the therapeutic actions of the mood stabilizers lithium and valproate, and that these mood stabilizers may regulate common target genes to exert their therapeutic effects. We have also identified a specific HDAC inhibitor

SAHA, as a potential novel mood stabilizing agent. Future studies will focus on determining common targets of these treatments, and the specific mechanisms, such as chromatin remodeling, through which these molecules are regulated in the hopes of identifying targets for the development of novel therapeutics in the treatment of BPD.

APPENDIX A:

AN INHIBITOR OF CASEIN KINASE 1ε/δ PARTIALLY NORMALIZES THE MANIC-LIKE BEHAVIORS OF THE *CLOCK*Δ19 MOUSE

Introduction

Bipolar disorder is a devastating disease that is characterized by periods of depression and mania. Though medications like lithium are effective in a number of people, the current mood stabilizers cause a number of significant side effects and do not alleviate symptoms in everyone with this disorder. Thus there is a need to develop better treatments. Several recent studies point towards disruptions in the circadian system as a strong contributing factor to the development of bipolar disorder (McClung 2007). Psychosocial therapies like Interpersonal and Social Rhythm Therapy (IPSRT) have demonstrated that regulation of the circadian cycle leads to improved patient outcomes in a shorter amount of time than traditional psychotherapy (Frank, Swartz et al. 2000). Recently, several drugs have been designed to inhibit Casein Kinase1 epsilon/delta (CK1ε/δ), critical proteins involved in the regulation of molecular rhythms. Circadian rhythms are controlled by a transcriptional/translational feedback loop which cycles over the course of 24 hours (Ko and Takahashi 2006). The CLOCK and BMAL1 proteins are the central transcriptional activators which regulate expression of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. These proteins then feedback and inhibit the activity of CLOCK/BMAL1. CK1ε/δ phosphorylates the PER1 and PER2 proteins leading to the recruitment of F-box protein β-TRCP which then targets the PER proteins for

degradation (Eide, Kang et al. 2005; Shirogane, Jin et al. 2005; Shanware, Hutchinson et al. 2011). This action helps regulate the timing of the molecular rhythms. Studies have found that the CK1ε/δ inhibitor, PF-670462 leads to a dose dependent phase delay in rhythms when administered chronically to wild type rats in a light/dark cycle (Badura, Swanson et al. 2007; Sprouse, Reynolds et al. 2010). These results were reproduced in diurnal Cynomolgus monkeys, suggesting that they would have the same effect on humans (Sprouse, Reynolds et al. 2009). Recently, Meng et al., 2010 reported that administration of PF-67042 could entrain mice with disrupted rhythms caused either by constant light or a mutation in the *Vipr2* gene (Meng, Maywood et al. 2010). These results suggest that PF-670462 or other CK1 ε/δ inhibitors could be used as a therapeutic tool to entrain rhythms of people with rhythm disruptions.

Our lab previously reported that mice with a mutation in the *Clock* gene (*Clock*Δ19 mice, described in King et al., 1997) have a behavioral profile which is strikingly similar to human mania (King, Vitaterna et al. 1997; Roybal, Theobold et al. 2007). This includes hyperactivity, decreased anxiety (i.e. increased exploratory drive), decreased depression-like behavior, and a hyperhedonic response to rewarding stimuli (McClung, Sidiropoulou et al. 2005; Roybal, Theobold et al. 2007). Lithium treatment is able to restore the majority of these abnormal behavioral phenotypes to wild type levels (Roybal, Theobold et al. 2007), suggesting that this mouse can be used to screen additional compounds which might be effective mood stabilizing drugs. We wanted to determine if a CK1 ε/δ inhibitor (CK01) would be able to restore normal behavior to the *Clock*Δ19 mouse. A positive result suggests that CK1ε/δ inhibitors may be effective compounds for rhythm and mood stabilization in bipolar subjects.

Materials and Methods

Animals and Housing *Clock*Δ19 mutant mice were created by *N*-ethyl-*N*-nitrosurea mutagenesis and produce a dominant-negative CLOCK protein defective in transcriptional activation activity as described (King, Vitaterna et al. 1997). For all experiments using *Clock*Δ19 mutants, 8 to 10 week old adult male mutant (*Clock/Clock;* Mut*)* and wild-type (+/+; WT) littermate controls on a mixed BALBc; C57BL/6 background were used. Mice were group housed in sets of 2-4 per cage on a 12:12 h light/dark cycle (lights on 6:00 a.m., lights off at 6:00 p.m) with food and water provided *ad libitum*.

Lithium Administration Lithium treated mice received 600mg/l of LiCl in drinking water for 10 days prior to behavioral testing, and throughout the course of the testing. This administration results in a stable serum concentration of lithium in the low therapeutic range for human patients (0.41±0.06 mmol/l), with little to no adverse health consequence (Roybal, Theobold et al. 2007).

CK01 Preparation and Administration CK01 was dissolved in 20% Sulfobutyl ether beta-cyclodextrin (SBE-β-cyclodextrin) in sterile water at 2 doses (17.8, 32.0 mg/kg) Approximately 4.8 microliters of 1N HCl was added per milligram of CK01 used to the vehicle solution. The CK01 solution was injected daily intraperitoneally for 10 days prior to behavioral testing, and daily injections continued throughout testing. The compound was administered at 6:30-7am (Zeitgeber time 1) each day.

Behavioral Assays

Locomotor Response to Novelty: Mice were placed into individual automated locomotor activity chambers that were equipped with infrared photobeams (San Diego Instruments, San Diego, CA). Activity measurements commenced upon the first beam break and were measured continuously and data was collected in five minute blocks over a period of two hours.

Elevated Plus Maze: The plus maze apparatus consisted of closed and open arms (all arms are 30 x 5 cm, with 25 cm tall walls on the closed arms). Mice were placed in the center of an elevated plus maze and the time spend in the open arms, closed arms, and center of the maze, along with the number of entries into the open and closed arms of the maze were determined by Ethovision 3.0 video tracking software (Noldus, Leesbrg, VA). Time spend on the open arm and percent of entries in to the open arm were used to determine anxiety-related behavior. The apparatus was cleaned and allowed to dry between every mouse.

Dark/Light Test: The dark/light apparatus is a 2 chambered box (25 cm x 26 cm for each side, Med Associates, St. Albans, VT), one side of which was kept dark, and the other side brightly lit by a fluorescent bulb at the top of the chamber. Mice were allowed to habituate to the dark side of the box for two minutes. Following the habituation period, the door between the compartments was opened and they were allowed to freely explore both sides of the apparatus for 10 minutes. Anxiety-like behavior was measured as the percent of time spent in the light side.

Forced Swim Test: Mice were placed in 4 liter Pyrex glass beakers were filled with 3 liters of water at 21-25°C for 6 minutes. All test sessions were recorded by a video camera from the side of the beakers. Water was changed between subjects. The video was analyzed and scored by an observer blind to the genotypes and treatment groups. After a 2 minute habituation time, latency to immobility was determined as the first cessation of movement. Total immobility was measured during the last four minutes of the test and was measured as time spent without movement except for a single limb paddling to maintain flotation.

Statistics

All data were analyzed by a two-way ANOVA followed by Bonferroni post-hoc tests, using GraphPad Prism 5 statistical software for Windows. All data are presented as means ± standard error of the mean with $p < 0.05$ considered statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Results

CK01 has no effect on general locomotor activity

To determine the effects of CK01 administration on manic-like behaviors, *Clock*Δ19 mice and WT littermates received 10 days of systemic CK01 at 2 doses (17.8 and 32.0 mg/kg) or SBE-β-cyclodextrin and were compared to *Clock*Δ19 and WT mice receiving lithium treatment following a battery of behavior behavioral testing. Mice were first assessed for their locomotor response to novelty. As reported previously (Roybal, Theobold et al. 2007). *Clock*Δ19 mice are hyperactive when compared to WT animals (Figure A-1), and CK01 administration had no significant effect on locomotor activity,

similar to mice receiving lithium treatment (Figure A-1). Because treatment had no effect on locomotor activity, effects on the other behavioral tests are due exclusively to changes in anxiety and depression related behavior.

CK01 normalizes the anxiolytic behavior of the *Clock*Δ19 mice

To examine the effects of CK01 administration on anxiety-related behavior, mice were subjected to two different measures: the elevated plus maze and dark/light test. In the elevated plus maze, the excess exploratory behavior of *Clock*Δ19 mice was rescued by the 32.0 mg/kg dose of CK01 as seen by a reduction in the amount of time spent in the open arms of the maze, similar to lithium treatment (Figure A-2a). The 17.8 mg/kg dose also caused a near-significant decrease in open arm time (Figure A-2a). In the dark/light test, CK01 also had anxiogenic effects similar to lithium treatment (Figure A-2b). CK01 treatment had no detectable effect on WT animals in any measure of anxiety-related behavior (Figure A-2a,b). A dose-dependent effect of CK01 treatment was observed, with a significant decrease in time spent in the light side of the light/dark box following administration of 17.8 mg/kg CK01 and a more robust decrease observed following 32.0 mg/kg CK01 treatment.

CK01 treatment partially normalizes the antidepressant effects of the *Clock*Δ19 mouse

In the forced swim test, *Clock*Δ19 mice displayed a significant decrease in depression-related behavior, as described previously (Figure A-3a; Roybal et al., 2007). Unlike lithium treatment, which has "anti-manic" effects on depression-related behavior by causing an increase in total immobile time (Figure A-3a), CK01 treatment had no

significant effect on immobile time. However, CK01 treatment did cause a significant decrease in latency to the first bout of immobility if *Clock*Δ19 mice without affecting WT animals suggesting a partial reversal of this phenotype (Figure A-3b).

Discussion

Our results show that CK01 treatment leads to a dose dependent reversal of the abnormal anxiolytic behaviors of the *Clock*Δ19 mouse. Furthermore, there was a partial reversal of the anti-depressant phenotypes. These behaviors constitute a profile of abnormal behavioral responses in the *Clock*Δ19 mouse which together represent a manic-like phenotype reminiscent of human bipolar disorder. Interestingly, CK01 treatment does not reverse the hyperactivity in a novel environment which is prominent in the *Clock*Δ19 mouse. Lithium treatment also does not reverse this phenotype and recent studies in our lab suggest that treatment with another mood stabilizing agent, valproate, also has no effect on this particular behavior. These results suggest that the hyperactivity in the *Clock*Δ19 mouse is controlled by a separate mechanism which is independent of the control of anxiety and mood-related behavior. This separation of mechanism is particularly relevant since amphetamine and other psychostimulant induced locomotor activity is often used as a model of mania. Indeed separate drugs may be needed to reverse specific endophenotypes of bipolar illness. Interestingly, a recent report found that PF-670462 does normalize amphetamine induced hyperactivity likely through a regulation of Darpp-32-PP1-GlurR1 signaling in the nucleus accumbens (NAc) (Li, Herrera et al. 2011). This suggests that CK1 inhibitors may be able to modulate certain

behavioral abnormalities via circadian clock stabilization and others through effects on modulating NAc output.

Previous studies have found that CK01 treatment leads to phase delays and a lengthening of the period of wild type animals while it entrains the rhythms of animals that are arrhythmic (Meng, Maywood et al. 2010). CK1δ inhibition leads to a daily enhancement of PER protein in the nucleus of the cell which presumably results from decreased degradation of the PER proteins or enhanced nuclear translocation. In *Clock*Δ19 mice, PER protein levels are very low and rhythms in a light dark cycle are sometimes weak (Vitaterna, Ko et al. 2006). Future studies will determine if CK01 is stabilizing the rhythms in these mice via increased PER protein concentrations in the suprachiasmatic nucleus.

It is unclear why there was only a partial reversal of depression-related behavior in the *Clock*Δ19 mice in the FST in that the latency to immobility was significantly altered but the total time immobile was not. It is possible that a higher dose or more chronic treatment paradigm would be sufficient to alter both parameters of this measure. It is also possible that CK1 proteins have a more prominent role in the control of anxiety. Nevertheless the results are promising and suggest that CK1 ε/δ inhibitors might be able to normalize both anxiety and mood-related phenotypes in human subjects.

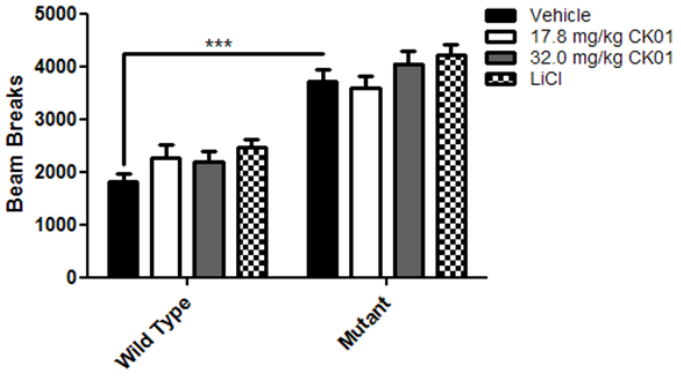


Figure A-1. CK01 administration has no effect on the locomotor response to novelty
Locomotor activity was measured in *Clock*Δ19 mice and wild type littermates for two hours following 10 days of CK01 or lithium administration. Analysis by two-way ANOVA revealed a significant main effect of genotype ($F_{(1,90)} = 145.94$, $p < 0.0001$). Bonferroni post-tests revealed that there was no significant effect of any treatment on locomotor response to novelty; n = 12-14 per group.

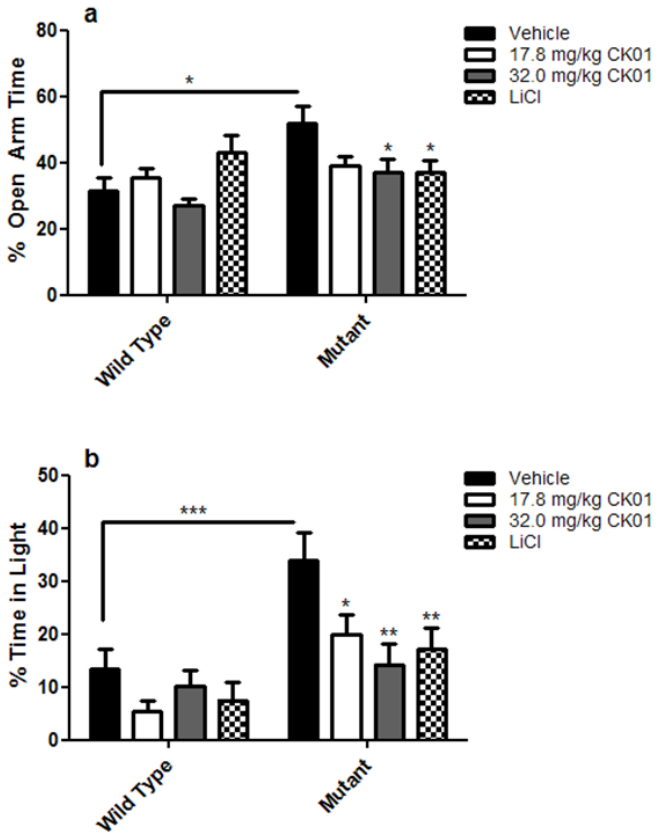


Figure A-2. CK01 administration normalized anxiety-related behavior in the ClockΔ19 mice. **A-B)** *Clock*Δ19 mice and wild type littermates were assessed for anxiety related behavior following CK01 and lithium administration in both the elevated plus maze (EPM,**A**) and dark/light test (**B**). Analysis by two-way ANOVA revealed that *Clock*Δ19 mice display previously reported decreased anxiety related behavior by spending more time in the open arms of the EPM (Main effect of genotype; $F_{(1,83)} = 6.39$, $p = 0.0134$) and light side of the dark/light box (Main effect of genotype; $F_{(1,85)} = 21.93$, $p<0.0001$). Bonferroni post tests revealed that 17.8 mg/kg CK01 treatment caused a reduction in open arm time that did not reach significance in the EPM (**A**) and a significant reduction in time spent in the light side of the dark/light test (**B**, $t = 2.553$, $p<0.05$). 32.0 mg/kg CK01 treatment in *Clock*Δ19 mice caused a significant decrease in EPM open arm time (**A**, $t = 2.553$, $p<0.05$) and time spent on the light side of the dark/light test (**B**, $t = 3.619$, $p<0.01$). As previously described, lithium had anti-manic effects on *Clock*Δ19 mice by restoring open-arm time in the EPM (**A**,$t = 2.747$, $p<0.05$) and time in the light side of the dark/light test (**B**, $t = 3.252$, $p<0.05$) to WT levels. CK01 treatment had no significant effect on anxiety-related behavior in wild type mice.

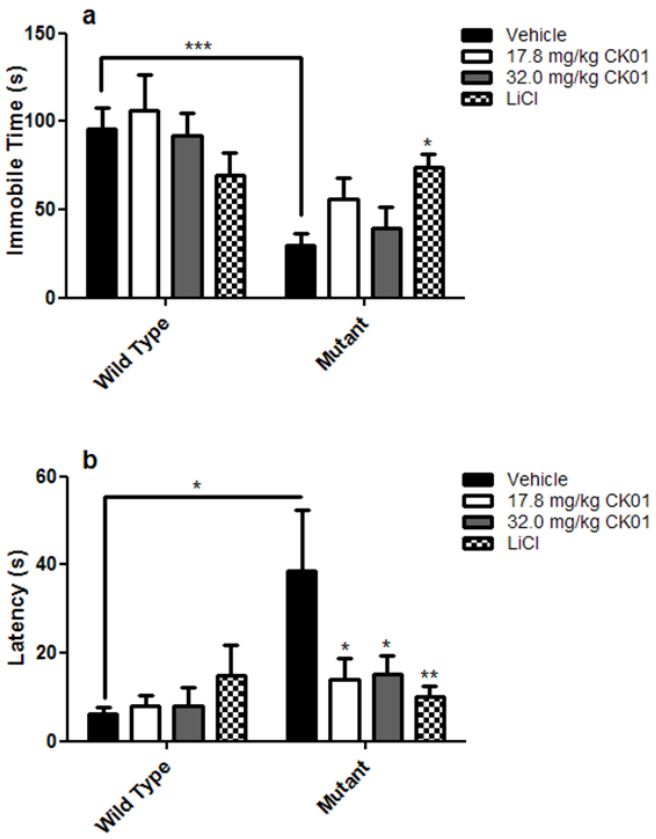


Figure A-3. CK01 administration has partial effects on *Clock*Δ19 depression-related behavior A) *Clock*Δ19 and wild type mice were assessed for depression-related behavior using the forced swim test following CK01 and lithium treatment. Analysis by two-way ANOVA showed that *Clock*Δ19 mice have decreased immobile time compared to wild type animals (Main effect of genotype; $F_{(1,88)} = 21.39$, $p<0.0001$). Bonferroni post-hoc tests displayed that lithium treatment had previously reported anti-manic effects on *Clock*Δ19 depression-related behavior by increasing total immobile time ($t = 2.52$, $p<0.05$). CK01 treatment had no detectable effect on either *Clock*Δ19 or WT immobile time. **B)** Latency to immobility was assessed following CK01 and lithium treatment in *Clock*Δ19 and wild type animals. *Clock*Δ19 mice have a significantly longer latency to immobility than wild type animals by two-way ANOVA (Main effect of genotype; $F_{(1,87)} = 6.30$, $p = 0.0140$). Bonferroni post-tests revealed a significant effect of treatment with 17.8 mg/kg CK01 ($t = 2.816$, $p<0.05$), 32.0 mg/kg CK01 ($t = 2.798$, $p<0.05$), and lithium ($t = 3.397$, $p<0.01$) treatment on latency to immobility in *Clock*Δ19 mice. Wild type animals were not significantly affected by treatment.

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