



Published in final edited form as:

*Mol Psychiatry*. 2014 March ; 19(3): 342–350. doi:10.1038/mp.2013.12.

## An important role for *Cholecystinin*, a CLOCK target gene, in the development and treatment of manic-like behaviors

Rachel N. Arey<sup>1</sup>, John F. Enwright III<sup>2,3</sup>, Sade M. Spencer<sup>1</sup>, Edgardo Falcon<sup>1</sup>, Angela R. Ozburn<sup>1,3</sup>, Subroto Ghose<sup>1</sup>, Carol Tamminga<sup>1</sup>, and Colleen A. McClung<sup>1,3,\*</sup>

<sup>1</sup>University of Texas Southwestern Medical Center, Department of Psychiatry, Dallas, TX 75390-9070

<sup>2</sup>Austin College, Department of Biology, Sherman, TX 75090

<sup>3</sup>University of Pittsburgh School of Medicine, Department of Psychiatry and Translational Neuroscience Program, Pittsburgh, PA 15219

### Abstract

Mice with a mutation in the *Clock* gene (*Clock*<sup>19</sup>) have been identified as a model of mania, 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 *Cholecystinin*(*Cck*) is a direct transcriptional target of CLOCK and levels of *Cck* are reduced in the ventral tegmental area (VTA) of *Clock*<sup>19</sup> mice. Selective knock-down of *Cck* expression via RNA interference (RNAi) in the VTA of wild type mice produces 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*<sup>19</sup> mice appears to be due to a lack of interaction with the histone methyltransferase, MLL1, resulting in decreased histone H3K4me3 and gene transcription, an effect reversed by lithium. Human postmortem tissue from bipolar subjects reveals a similar increase in *Cck* expression in the VTA with mood stabilizer treatment. These studies identify a key role for *Cck* in the development and treatment of mania, and describe some of the molecular mechanisms by which lithium may act as an effective anti-manic agent.

### Keywords

Bipolar disorder; dopamine; lithium; chromatin structure; gene expression

### Introduction

Bipolar disorder (BPD) is a chronic psychiatric disease that afflicts approximately 1–3% of the United States population.<sup>1</sup> The underlying cause of BPD is unknown, though there is a

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: [http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

\*Author to whom correspondence should be addressed, Colleen A. McClung, Ph.D., University of Pittsburgh School of Medicine, Department of Psychiatry, 450 Technology Drive, Suite 223, Pittsburgh, PA 15219, (412) 624-5547 phone, mcclungca@upmc.edu.

The authors declare no conflict of interest.

growing body of evidence linking disruptions in circadian rhythms with the disease.<sup>2</sup> Within the suprachiasmatic nucleus (SCN) and other regions, circadian rhythms are controlled by the molecular clockwork, which is comprised of a series of autoregulatory transcriptional-translational feedback loops. The transcription factors CLOCK and BMAL1 heterodimerize and activate transcription of target genes, including the *Period (Per)* and *Cryptochrome (Cry)* genes which act to inhibit the activity of the CLOCK/BMAL1 complex.<sup>3</sup> Recent human genetic studies have linked elements of the molecular clockwork to BPD. Polymorphisms in CLOCK and other circadian genes are associated with various aspects of bipolar disorder.<sup>4–12</sup> In addition, rhythm disruptions and sleep disturbances are common in BPD and often precipitate manic or depressive episodes.<sup>13, 14</sup>

Mice bearing a dominant negative mutation (*Clock*<sup>19</sup>) in the *Clock* gene<sup>15</sup> have a behavioral profile which is very similar to human mania.<sup>16, 17</sup> These mice exhibit hyperactivity, decreased anxiety-related and depression-related behavior, and increased preference for rewarding stimuli.<sup>16, 17</sup> Furthermore, the majority of these behavioral abnormalities can be reversed with chronic lithium treatment.<sup>17</sup> Previous studies from our group have identified an important role for the VTA in the development of this manic-like phenotype. When CLOCK levels are decreased specifically in the VTA of wild type (WT) animals, behaviors similar to *Clock*<sup>19</sup> mice, including hyperactivity and decreased anxiety are induced.<sup>18</sup> Conversely, when a functional CLOCK protein is expressed only in the VTA of *Clock*<sup>19</sup> mice, locomotor activity and anxiety-related behavior are restored to wild type levels.<sup>17</sup> Interestingly, the *Clock*<sup>19</sup> mice also have increased firing and bursting of VTA dopamine neurons which is reversed with chronic lithium treatment.<sup>19</sup>

Microarray analysis of VTA tissue from *Clock*<sup>19</sup> mice and WT littermates revealed altered transcription of many genes involved in dopaminergic transmission.<sup>16</sup> One of the genes identified as significantly down regulated was the neuropeptide transmitter, *cholecystokinin (CCK)*.<sup>16</sup> The sulphated carboxy terminal octapeptide, CCK-8S, is the most commonly expressed form in the brain, with larger forms expressed in the gut. The primary action of CCK in the brain is thought to be mediated through the CCK<sub>B</sub> receptor, which has been shown in cultured striatal neurons to increase intracellular calcium levels.<sup>20</sup> Within the VTA and the substantia nigra, CCK is highly co-localized with dopaminergic neurons that project to the nucleus accumbens (NAc), with 40–80% of the cells co-expressing dopamine and CCK.<sup>21, 22</sup> At VTA dopaminergic terminals, CCK is co-released with dopamine, specifically upon burst firing.<sup>23</sup> CCK acts as a negative modulator of dopaminergic transmission *in vivo*, as infusions of CCK-8S into the NAc inhibit K<sup>+</sup>-stimulated dopamine release and reduce extracellular dopamine concentrations.<sup>24</sup> Behavioral studies using CCK agonists and antagonists find that increased CCK results in increased anxiety and depression-like behavior while CCK receptor blockade is anxiolytic and antidepressant.<sup>25</sup> Thus, it is possible that the decreased CCK levels in the VTA of the *Clock*<sup>19</sup> mice are responsible for their overall manic-like phenotype. Here we wanted to determine the role of CCK in the development of manic-like symptoms in the *Clock*<sup>19</sup> mutant mice, as well as the reversal of these phenotypes with lithium treatment. Moreover, we wanted to know the mechanism by which CLOCK regulates CCK expression.

## Materials and Methods

### Animals

*Clock*<sup>19</sup> mutant mice were created by *N*-ethyl-*N*-nitrosurea mutagenesis and produce a dominant-negative CLOCK protein defective in transcriptional activation activity as described.<sup>26</sup> For all experiments using *Clock*<sup>19</sup> mutants, 8 to 16 week old adult male mutant (*Clock*<sup>19</sup>; 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. All behavioral and molecular assays were performed between ZT 7–11.

### 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 consequences.<sup>17</sup>

### Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed according to methods described previously.<sup>27, 28</sup> Additional details are in the Supplemental Material.

### Behavioral Assays

The locomotor response to novelty, Elevated Plus Maze, Dark/Light test and Forced Swim test all utilized standard protocols and were performed as described previously.<sup>18</sup> Additional details are in the Supplemental Material.

### 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 Ct method as previously described.<sup>29</sup>

### Reporter and Expression Plasmids

The wild-type *Cck* luciferase reporter was described previously.<sup>27</sup> Additional details are in the Supplemental Material.

### Luciferase Assays

Cell culture and performance of the luciferase assay were carried out as described previously.<sup>27</sup> Additional details are in the Supplemental Material.

### **Construction of AAV- Cck-shRNA and Virus Purification**

A small hairpin RNA (shRNA) directed against *Cck* was designed using previously published criteria.<sup>18</sup> For the *Cck*shRNA, a 15 base pair sequence in the coding region of the *Cck* gene (5'-CTTGAGCGGTTTCGG-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) were 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. Additional details are in the Supplemental Material.

### **Laser Capture Microdissection**

Laser capture microdissection (LCM) to assess levels of in vivo *Cck* knockdown were performed as in previous studies.<sup>18</sup> Additional details are in the Supplemental Material.

### **Stereotaxic Surgery**

Surgery was performed as described previously.<sup>18</sup> Additional details are in the Supplemental Material.

### **Immunohistochemistry and validations of injections and infections**

Validation of injections was performed as published previously.<sup>18</sup> Additional details are in the Supplemental Material.

### **Subject selection and tissue acquisition**

Human brain tissue from depression and control cases was obtained from the Dallas Brain Collection.<sup>30</sup> The tissue was collected only after acquiring consent from the next of kin along with permission to review medical records and to conduct a telephone interview with a primary caregiver. All clinical information on each case was evaluated by at least three research psychiatrists and diagnoses made using DSM IV criteria. Blood screens for drugs of abuse, alcohol and prescription medications were conducted on each case. Cases were excluded when there was a known history of neurological disorders or of an axis I psychiatric condition other than major depression. The method of collection and storage of human brain tissue is approved by the Institutional Review Board of the University of Texas Southwestern Medical Center.

### **Human tissue preparation**

In each case, cerebral hemispheres were cut coronally into 1–1.5 cm blocks and the midbrain was cut into 1–1.5 cm blocks in a plane perpendicular to its long axis as previously described<sup>31</sup>. Additional details are in the Supplemental Material.

### **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 or more groups. When more than one factor was examined simultaneously, two-way ANOVAs were performed followed by Bonferroni post hoc tests. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . In all figures error bars show S.E.M.

## Results

### CCK is a direct CLOCK target gene in the VTA

To determine if the previously observed decrease in *Cckm* RNA levels was a direct or indirect result of the *Clock*<sup>-19</sup> mutation, we measured CLOCK binding at the *Cck* promoter. The *Cck* promoter contains an E-box element (CANNTG) that is part of a 110 bp proximal promoter region that is over 80% conserved from mouse to human.<sup>32</sup> In addition to the E-box, there is also a GC-rich region, CRE/TRE site, and putative TATA box (Figure 1a). To assess potential CLOCK binding at the *Cck* promoter, chromatin immunoprecipitation (ChIP) assays were carried out on VTA-containing tissue from WT and *Clock*<sup>-19</sup> littermates. CLOCK was enriched at the *Cck* promoter above background in both genotypes with no significant difference between the two (Figure 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 1c). When the E-box was mutated, the ability of transfected CLOCK/BMAL1 to induce *Cck*-luc reporter activity was abolished (Figure 1c). These results demonstrate that CLOCK acts as a direct, positive regulator of the *Cck* gene in an E-box dependent manner. Furthermore, the decrease in *Cck* gene expression observed in the *Clock*<sup>-19</sup> mice is not due to a loss of CLOCK binding at the promoter, but rather a loss of function of the CLOCK protein as a transcriptional regulator.

### *Cck* expression in the *Clock*<sup>-19</sup> mice is reduced due to a lack of MLL1 binding at the promoter

Previous *in vitro* studies have found that CLOCK activates transcription *via* interactions with histone methyltransferase mixed lineage leukemia 1 (MLL1), which then causes trimethylation of histone H3 at lysine 4 (H3K4me3), and allows for transcriptional activation of CLOCK target genes.<sup>33</sup> To determine if this is the case *in vivo* at the *Cck* promoter, ChIP assays were carried out using an antibody against MLL1. We found a significant decrease in both MLL1 and H3K4me3 levels at the *Cck* promoter in the midbrain of the *Clock*<sup>-19</sup> mice compared to WT animals (Figure 1d, e). Thus, *Cck* levels are likely reduced in the *Clock*<sup>-19</sup> mice due to the inability of MLL1 to bind CLOCK<sup>-19</sup> protein, leading to a decrease in H3K4me3.

### Knock-down of CCK in the VTA of WT animals results in a manic-like phenotype

To determine if a loss of *Cck* expression in the VTA is sufficient to induce manic-like behaviors, an AAV-shRNA directed specifically against *Cck* was generated (AAV-Cck-shRNA). An AAV-scrambled sequence (AAV-Scr) that does not match any known gene was used as a control.<sup>18</sup> Stereotaxic injection of AAV-Cck-shRNA into the VTA of

C57BL6J mice resulted in a significant (~6-fold) decrease in *Cck* mRNA levels after 3 weeks incubation compared to AAV-Scr controls (Supplemental Figure 1). Following the confirmation of knock-down, a separate cohort of mice injected with AAV-Cck-shRNA or AAV-Scr were then subjected to a battery of behavioral tests. When exposed to a novel environment, mice injected with AAV-Cck-shRNA in the VTA were significantly hyperactive compared to those injected with AAV-Scr over the course of two hours (Figure 2a). Mice were then subjected to two anxiety-related behavioral tests: the elevated plus maze and light/dark box. AAV-Cck-shRNA injected mice spent significantly more time in the open arms of the elevated plus maze and in the light side of the light/dark box, suggesting that they have decreased anxiety (Figure 2b, c). We then determined the effects of *Cck* knock-down on depression-related behavior by subjecting animals to the Porsolt forced swim test. Decreased immobility time and an increase in latency to first bout of immobility was observed in mice injected with AAV-Cck-shRNA in the VTA compared to AAV-Scr control mice (Figure 2d, 2e). Together these results indicate that a knock-down of *Cck* in the VTA decreases anxiety and depression-related behavior.

### Lithium selectively restores *Cck* expression levels in *Clock*<sup>19</sup> mice

Since we were able to determine that a decrease in *Cck* is sufficient to induce manic-like behaviors, and lithium treatment rescues *Clock*<sup>19</sup> mutant behavior<sup>17</sup>, we investigated whether *Cck* levels were altered by lithium treatment. mRNA was isolated from the VTA of *Clock*<sup>19</sup> and littermate control mice receiving either chronic lithium (600 mg/L in drinking water) or normal drinking water, and qPCR was performed to assess *Cck* mRNA levels. Consistent with our previous microarray results<sup>16</sup>, *Cck* expression was decreased in *Clock*<sup>19</sup> mice relative to WT littermates (Figure 3a). When *Clock*<sup>19</sup> mice were administered lithium, *Cck* mRNA levels were restored to near WT levels (Figure 3a). Interestingly, lithium treatment had no detectable effect on WT *Cck* mRNA levels (Figure 3a) suggesting a specific effect in mice with a manic-like phenotype.

### Regulation of chromatin structure at the *Cck* promoter by lithium treatment

We wanted to begin to understand the molecular mechanisms by which lithium treatment regulates the *Cck* gene. ChIP assays were carried out using an antibody against H3K4me3 in *Clock*<sup>19</sup> mice receiving either water or lithium. Lithium treatment in *Clock*<sup>19</sup> mice led to an increase of H3K4me3 at the *Cck* promoter (Figure 3b) and a trend toward increased levels of MLL1 at the *Cck* promoter in *Clock*<sup>19</sup> mice, though this did not reach significance (Figure 3c). This suggests that other histone methyltransferases are likely involved. Lithium treatment had no significant effect on H3K4me3 or MLL1 levels at the *Cck* promoter in WT animals (Supplemental figure 2). We then wanted to determine whether lithium could regulate histone acetylation at the *Cck* promoter. ChIP assays were carried out using antibodies against AcH3 and AcH4 in *Clock*<sup>19</sup> mice and WT controls receiving either water or lithium (Figure 3d, e). Lithium treatment caused a selective increase in levels of both AcH3 and AcH4 at the *Cck* promoter in *Clock*<sup>19</sup> mice, while levels of AcH3 and AcH4 are not significantly affected in WT mice (Figure 3d, e). To determine how lithium treatment results in recruitment of chromatin remodeling enzymes to the *Cck* promoter, we assessed whether there were any changes in mutant CLOCK protein binding to the *Cck* promoter. A significant reduction in CLOCK<sup>19</sup> protein at the *Cck*



promoter was observed in *Clock*<sup>-/-</sup> mice receiving lithium treatment, suggesting that lithium causes another DNA-binding protein to compete with CLOCK<sup>-/-</sup> and this results in recruitment of chromatin remodeling enzymes that rescue expression (Figure 3f).

### **Cck levels are altered in human subjects with bipolar disorder following treatment**

Because a restoration of *Cck* levels in the VTA seems to correlate with the therapeutic actions of lithium in the *Clock*<sup>-/-</sup> mice, we wanted 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 at the time of death. Information on individual human postmortem samples is detailed in Tables S2, S3, and S4. qPCR was performed to assess *Cck* mRNA levels. While there was no detectable difference in *Cck* levels in the VTA between control subjects and BPD patients not receiving medication (Figure 4), interestingly, BPD patients receiving pharmacological treatment had a significant increase in *Cck* levels in the VTA (Figure 4).

### **Increased Cck levels in the VTA are required for the therapeutic effects of lithium in Clock<sup>-/-</sup> mice**

We injected AAV-Cck-shRNA or AAV-Scr into the VTA of *Clock*<sup>-/-</sup> mice. The mice then received chronic lithium treatment or normal drinking water to determine if lithium could still rescue *Clock*<sup>-/-</sup> anxiety and depression related behavior when it was unable to restore proper *Cck* levels. *Clock*<sup>-/-</sup> mice injected with AAV-Cck-shRNA were hyperactive compared to AAV-Scr controls (Figure 5a). Consistent with previous findings<sup>17</sup>, this lithium treatment paradigm had no detectable effect on locomotor activity in animals injected with either virus (Figure 5a). The mice were then subjected to the elevated plus maze and dark/light box to measure anxiety-related behavior. Importantly, lithium had no effect on the *Clock*<sup>-/-</sup> mice injected with the AAV-Cck-shRNA in the VTA while it was able to normalize the anxiety-related behavior in the mice injected with the AAV-Scr as measured by the percent open arm time of the EPM (Figure 5b) and the percent time in the light in the dark/light box (Figure 5c). Mice were then subjected to the forced swim test to measure depression-related behavior. Similar to what was observed in the anxiety-related behavioral tests, lithium was successful in reversing the time spent immobile and the latency to immobility in the *Clock*<sup>-/-</sup> mice injected with AAV-Scr but it had no effect on the mice injected with AAV-Cck-shRNA in the VTA (Figure 5d, e). These results demonstrate that an increase in *Cck* levels in the VTA is necessary for lithium to have therapeutic effects.

## **Discussion**

Our results identify the peptide neurotransmitter *Cck* as a novel and direct transcriptional 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. It is likely that CLOCK also contributes to the strong circadian rhythm in *Cck* expression.<sup>34, 35</sup> As expected by the sequence of the CLOCK<sup>-/-</sup> protein, this protein can still bind to the *Cck* promoter but cannot activate transcription. We find that levels of H3K4me3, a histone modification associated with transcriptional activation, are decreased at the *Cck* promoter when compared to WT mice. This is likely due 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*<sup>-/-</sup> mice relative to WT littermates. Though this lack of association was thought to underlie the dominant negative function of CLOCK<sup>-/-</sup> based on findings in cell culture at other gene promoters, we provide the first evidence to suggest that this is indeed the case in the brain, and that this interaction is important for regulation at the *Cck* promoter.

Furthermore, we have determined that a knock-down of *Cck* in the VTA produces a manic-like phenotype similar to the *Clock*<sup>-/-</sup> mice. AAV-mediated knock-down of *Cck* specifically in the VTA results in hyperactivity, decreased anxiety-related behavior, and a decrease in depression-related behavior. These results correspond well to previous studies with systemic administration of CCK<sub>B</sub> receptor antagonists, which are anxiolytic and antidepressant.<sup>36,37</sup> Since these changes in behavior are very similar to those seen in the *Clock*<sup>-/-</sup> mice, the “manic-like” phenotype of these mice may ultimately be due to a decrease in *Cck* levels in the VTA. Interestingly, the behavioral effects of *Cck* knock-down differ somewhat from those observed in mice that have AAV-mediated knock-down of CLOCK specifically in the VTA, which results in hyperactivity and decreased anxiety, but also results in an increase in depression related behavior when compared to control animals.<sup>18</sup> Given the nature of viral mediated gene transfer and the fact that this was a gene knock-down experiment rather than an overexpression of the CLOCK<sup>-/-</sup> protein, there are a number of possibilities that might explain this discrepancy and it will be interesting to explore these in future studies.

Chronic lithium administration results in a restoration of *Cck* mRNA to near WT levels in the VTA of the *Clock*<sup>-/-</sup> mice with no detectable effect on WT *Cck* levels. This finding makes *Cck* a particularly promising target as lithium treatment selectively rescues the *Clock*<sup>-/-</sup> mouse behavior while having little effect on WT animals.<sup>17</sup> 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 healthy controls.<sup>38,39</sup> Therefore, the selective regulation of *Cck* by lithium may be of particular therapeutic relevance.

Indeed, we find an increase in *Cck* levels in human BPD patients receiving pharmacological treatments. Unlike *Clock*<sup>-/-</sup> mice which have a large decrease in *Cck* in the VTA there was no detectable difference in *Cck* expression between normal controls and BPD patients not receiving medications. 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*<sup>-/-</sup> mice, and the mood state of the BPD patients at the time of tissue collection was not known.

Lithium treatment leads to a restoration of H3K4me3 levels at the *Cck* promoter in *Clock*<sup>-/-</sup> mice without affecting WT animals. This rescue of H3K4me3 is not accompanied by a significant increase in MLL1 at the *Cck* promoter, indicating that factors other than MLL1 likely contribute to lithium’s effects on H3K4me3 in the *Clock*<sup>-/-</sup> mice. In addition to an increase in H3K4me3, an increase in AcH3 and AcH4 was observed at the *Cck* promoter in the *Clock*<sup>-/-</sup> mice following lithium treatment. It has recently become clear that H3K4me3 and histone acetylation are linked, and there is a large amount of crosstalk between proteins that deposit these modifications on histone tails.<sup>40–43</sup> Interestingly there is no decrease in



levels of AcH3 or AcH4 at the *Cck* promoter in untreated *Clock*<sup>-19</sup> mice, despite the decrease in H3K4me3. This is probably due to the fact that CLOCK<sup>-19</sup>, though unable to recruit MLL1, still retains its histone acetyltransferase (HAT) activity.<sup>33, 44, 45</sup> Interestingly, this increase in histone acetylation and methylation specifically occurs in the context of a disease state (i.e. a manic-like phenotype) and does not occur in wild type animals. This might explain why previous animal studies have not identified lithium as a drug that alters chromatin structure. The increase in AcH3 and AcH4 following lithium treatment is likely due to the recruitment of HATs rather than an inhibition of HDAC proteins which is an activity that may underlie the efficacy of valproic acid as a mood stabilizing drug.<sup>46</sup> Lithium treatment leads to a decrease in CLOCK<sup>-19</sup> levels at the *Cck* promoter in the *Clock*<sup>-19</sup> mice, suggesting that another protein or transcriptional-activation complex is competing with CLOCK<sup>-19</sup> for *Cck* promoter occupancy. Future studies will determine what this factor is and how lithium acts to bring this factor to the *Cck* promoter.

The increase in *Cck* produced by lithium is necessary for its therapeutic effects in the *Clock*<sup>-19</sup> mice, as *Clock*<sup>-19</sup> mice injected with AAV-Cck-shRNA into the VTA no longer respond to lithium treatment. Interestingly, *Cck* knock-down in wild type mice results in a hyperactive locomotor response to novelty, and the *Clock*<sup>-19</sup> mice are hyperactive, however, lithium treatment has no effect on locomotor activity in the *Clock*<sup>-19</sup> mice either with or without injection of the AAV-Cck-shRNA. These results clearly separate hyperlocomotor activity from measures of anxiety and depression-related behavior, giving us further confidence in the interpretation of these measures. Moreover, it suggests a separate mechanism by which these processes are regulated.

In conclusion, we have identified a novel CLOCK target gene, *Cck*, which likely contributes to the phenotype of the *Clock*<sup>-19</sup> mice since a decrease in *Cck* expression in the VTA results in a manic-like phenotype. The decrease in *Cck* transcription in the *Clock*<sup>-19</sup> mice is likely due to the inability of CLOCK<sup>-19</sup> to interact with the histone methyltransferase, MLL1, which results in a decrease of H3K4me3 at the *Cck* promoter. In addition, we have determined that an increase in *Cck* mRNA levels in the VTA is necessary for lithium's therapeutic actions in the *Clock*<sup>-19</sup> mice. Lithium regulates the *Cck* gene via an increase in H3K4me3, AcH3, and AcH4 at the *Cck* promoter in the *Clock*<sup>-19</sup> mice. The precise mechanism by which these histone modifications are increased at the *Cck* promoter following lithium treatment will be the subject of future studies. The hope is that these studies 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We are grateful to Dr. Joe Takahashi for the *Clock*<sup>-19</sup> mice. We thank Elizabeth Gordon and Ariel Ketcherside for assistance with mouse husbandry and genotyping, Dr. Shari Birnbaum and Ami Petterson for assistance with behavioral testing, and Dr. Shibani Mukherjee for assistance with AAV construction and design. We also thank Dr.

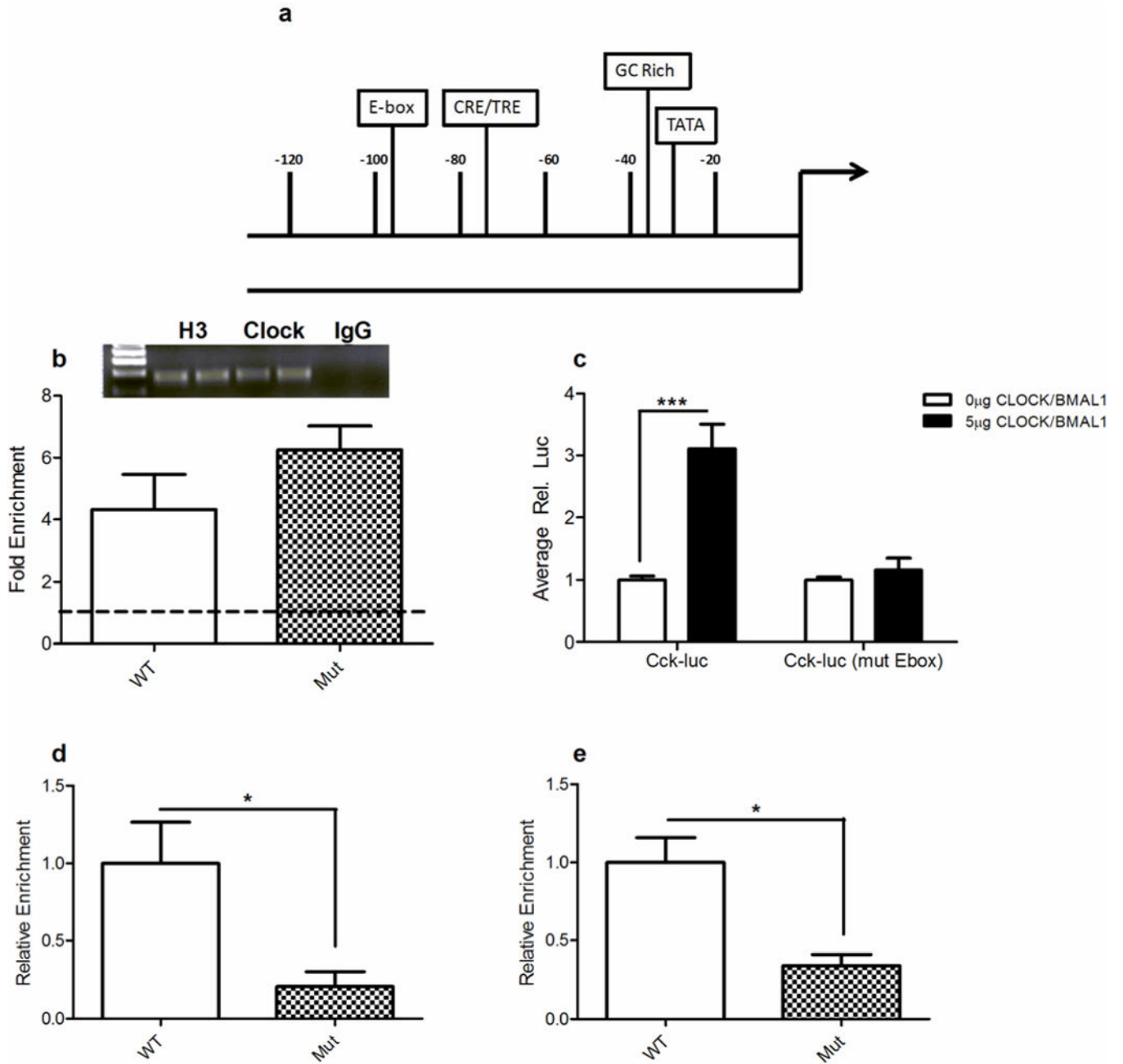
Syann Lee and Dr. Joel Elmquist for assistance with laser capture equipment. We also thank Dr. R. Jude Samulski and the UNC Gene Therapy Vector Core for assistance with AAV preparation. This study was funded by The McKnight Endowment Fund for Neuroscience, The Brain & Behavior Research Foundation (NARSAD), and the NIMH (MH082876).

## References

1. Kupfer DJ, Angst J, Berk M, Dickerson F, Frangou S, Frank E, et al. Advances in bipolar disorder: selected sessions from the 2011 International Conference on Bipolar Disorder. *Ann N Y Acad Sci.* 2011; 1242(1):1–25. [PubMed: 22191553]
2. McClung CA. Circadian genes, rhythms and the biology of mood disorders. *Pharmacol Ther.* 2007; 114(2):222–232. [PubMed: 17395264]
3. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. *Hum Mol Genet.* 2006; 15:R271–277. Spec No 2. [PubMed: 16987893]
4. Lamont EW, Coutu DL, Cermakian N, Boivin DB. Circadian rhythms and clock genes in psychotic disorders. *Isr J Psychiatry Relat Sci.* 2010; 47(1):27–35. [PubMed: 20686197]
5. Benedetti F, Dallaspezia S, Fulgosi MC, Lorenzi C, Serretti A, Barbini B, et al. Actimetric evidence that CLOCK 3111 T/C SNP influences sleep and activity patterns in patients affected by bipolar depression. *Am J Med Genet B Neuropsychiatr Genet.* 2007; 144B(5):631–635. [PubMed: 17221848]
6. Serretti A, Benedetti F, Mandelli L, Lorenzi C, Pirovano A, Colombo C, et al. Genetic dissection of psychopathological symptoms: insomnia in mood disorders and CLOCK gene polymorphism. *Am J Med Genet B Neuropsychiatr Genet.* 2003; 121B(1):35–38. [PubMed: 12898572]
7. Soria V, Martinez-Amoros E, Escaramis G, Valero J, Perez-Egea R, Garcia C, et al. Differential association of circadian genes with mood disorders: CRY1 and NPAS2 are associated with unipolar major depression and CLOCK and VIP with bipolar disorder. *Neuropsychopharmacology.* 2010; 35(6):1279–1289. [PubMed: 20072116]
8. Mansour HA, Wood J, Logue T, Chowdari KV, Dayal M, Kupfer DJ, et al. Association study of eight circadian genes with bipolar I disorder, schizoaffective disorder and schizophrenia. *Genes Brain Behav.* 2006; 5(2):150–157. [PubMed: 16507006]
9. Mansour HA, Talkowski ME, Wood J, Chowdari KV, McClain L, Prasad K, et al. Association study of 21 circadian genes with bipolar I disorder, schizoaffective disorder, and schizophrenia. *Bipolar Disord.* 2009; 11(7):701–710. [PubMed: 19839995]
10. Kripke DF, Nievergelt CM, Joo E, Shekhtman T, Kelsoe JR. Circadian polymorphisms associated with affective disorders. *J Circadian Rhythms.* 2009; 7:2. [PubMed: 19166596]
11. Nievergelt CM, Kripke DF, Barrett TB, Burg E, Remick RA, Sadovnick AD, et al. Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet.* 2006; 141B(3):234–241. [PubMed: 16528748]
12. Sjöholm LK, Backlund L, Cheteh EH, Ek IR, Frisen L, Schalling M, et al. CRY2 is associated with rapid cycling in bipolar disorder patients. *PLoS One.* 2010; 5(9):e12632. [PubMed: 20856823]
13. Lamont EW, Legault-Coutu D, Cermakian N, Boivin DB. The role of circadian clock genes in mental disorders. *Dialogues Clin Neurosci.* 2007; 9(3):333–342. [PubMed: 17969870]
14. Harvey AG. Sleep and circadian functioning: critical mechanisms in the mood disorders? *Annu Rev Clin Psychol.* 2011; 7:297–319. [PubMed: 21166537]
15. Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science.* 1994; 264(5159):719–725. [PubMed: 8171325]
16. McClung CA, Sidiropoulou K, Vitaterna M, Takahashi JS, White FJ, Cooper DC, et al. Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proc Natl Acad Sci U S A.* 2005; 102(26):9377–9381. [PubMed: 15967985]
17. Roybal K, Theobald D, Graham A, DiNieri JA, Russo SJ, Krishnan V, et al. Mania-like behavior induced by disruption of CLOCK. *Proc Natl Acad Sci U S A.* 2007; 104(15):6406–6411. [PubMed: 17379666]

18. Mukherjee S, Coque L, Cao JL, Kumar J, Chakravarty S, Asaithamby A, et al. Knockdown of Clock in the Ventral Tegmental Area Through RNA Interference Results in a Mixed State of Mania and Depression-Like Behavior. *Biol Psychiatry*. 2010
19. Coque L, Mukherjee S, Cao JL, Spencer S, Marvin M, Falcon E, et al. Specific role of VTA dopamine neuronal firing rates and morphology in the reversal of anxiety-related, but not depression-related behavior in the ClockDelta19 mouse model of mania. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2011; 36(7):1478–1488. [PubMed: 21430648]
20. Miyoshi R, Kito S, Nomoto T. Cholecystokinin increases intracellular Ca<sup>2+</sup> concentration in cultured striatal neurons. *Neuropeptides*. 1991; 18(3):115–119. [PubMed: 2067597]
21. Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, Markey K. Evidence for coexistence of dopamine and CCK in meso-limbic neurones. *Nature*. 1980; 285(5765):476–478. [PubMed: 6105617]
22. Lanca AJ, De Cabo C, Arifuzzaman AI, Vaccarino FJ. Cholecystokinergic innervation of nucleus accumbens subregions. *Peptides*. 1998; 19(5):859–868. [PubMed: 9663451]
23. Ghijsen WE, Leenders AG, Wiegant VM. Regulation of cholecystokinin release from central nerve terminals. *Peptides*. 2001; 22(8):1213–1221. [PubMed: 11457513]
24. Voigt MM, Wang RY. In vivo release of dopamine in the nucleus accumbens of the rat: modulation by cholecystokinin. *Brain Res*. 1984; 296(1):189–193. [PubMed: 6324955]
25. Rotzinger S, Lovejoy DA, Tan LA. Behavioral effects of neuropeptides in rodent models of depression and anxiety. *Peptides*. 2010; 31(4):736–756. [PubMed: 20026211]
26. King DP, Vitaterna MH, Chang AM, Dove WF, Pinto LH, Turek FW, et al. The Mouse Clock Mutation Behaves as an Antimorph and Maps Within the W(19H) Deletion, Distal of Kit. *Genetics*. 1997; 146(3):1049–1060. [PubMed: 9215907]
27. Enwright JF 3rd, Wald M, Paddock M, Hoffman E, Arey R, Edwards S, et al. DeltaFosB indirectly regulates Cck promoter activity. *Brain Res*. 2010; 1329:10–20. [PubMed: 20226774]
28. Tsankova NM, Kumar A, Nestler EJ. Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. *J Neurosci*. 2004; 24(24):5603–5610. [PubMed: 15201333]
29. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci*. 2006; 9(4):519–525. [PubMed: 16501568]
30. Stan AD, Ghose S, Gao XM, Roberts RC, Lewis-Amezcuea K, Hatanpaa KJ, et al. Human postmortem tissue: what quality markers matter? *Brain Res*. 2006; 1123(1):1–11. [PubMed: 17045977]
31. Ghose S, Crook JM, Bartus CL, Sherman TG, Herman MM, Hyde TM, et al. Metabotropic glutamate receptor 2 and 3 gene expression in the human prefrontal cortex and mesencephalon in schizophrenia. *Int J Neurosci*. 2008; 118(11):1609–1627. [PubMed: 18853337]
32. Hansen TV. Cholecystokinin gene transcription: promoter elements, transcription factors and signaling pathways. *Peptides*. 2001; 22(8):1201–1211. [PubMed: 11457512]
33. Katada S, Sassone-Corsi P. The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol*. 2010; 17(12):1414–1421. [PubMed: 21113167]
34. Weber M, Lauterburg T, Tobler I, Burgunder JM. Circadian patterns of neurotransmitter related gene expression in motor regions of the rat brain. *Neuroscience letters*. 2004; 358(1):17–20. [PubMed: 15016424]
35. Schade R, Vick K, Ott T, Sohr R, Pfister C, Bellach J, et al. Circadian rhythms of dopamine and cholecystokinin in nucleus accumbens and striatum of rats--influence on dopaminergic stimulation. *Chronobiol Int*. 1995; 12(2):87–99. [PubMed: 8653803]
36. Hernando F, Fuentes JA, Roques BP, Ruiz-Gayo M. The CCKB receptor antagonist, L-365,260, elicits antidepressant-type effects in the forced-swim test in mice. *Eur J Pharmacol*. 1994; 261(3):257–263. [PubMed: 7813546]
37. Hughes J, Boden P, Costall B, Domeney A, Kelly E, Horwell DC, et al. Development of a class of selective cholecystokinin type B receptor antagonists having potent anxiolytic activity. *Proc Natl Acad Sci U S A*. 1990; 87(17):6728–6732. [PubMed: 1975695]

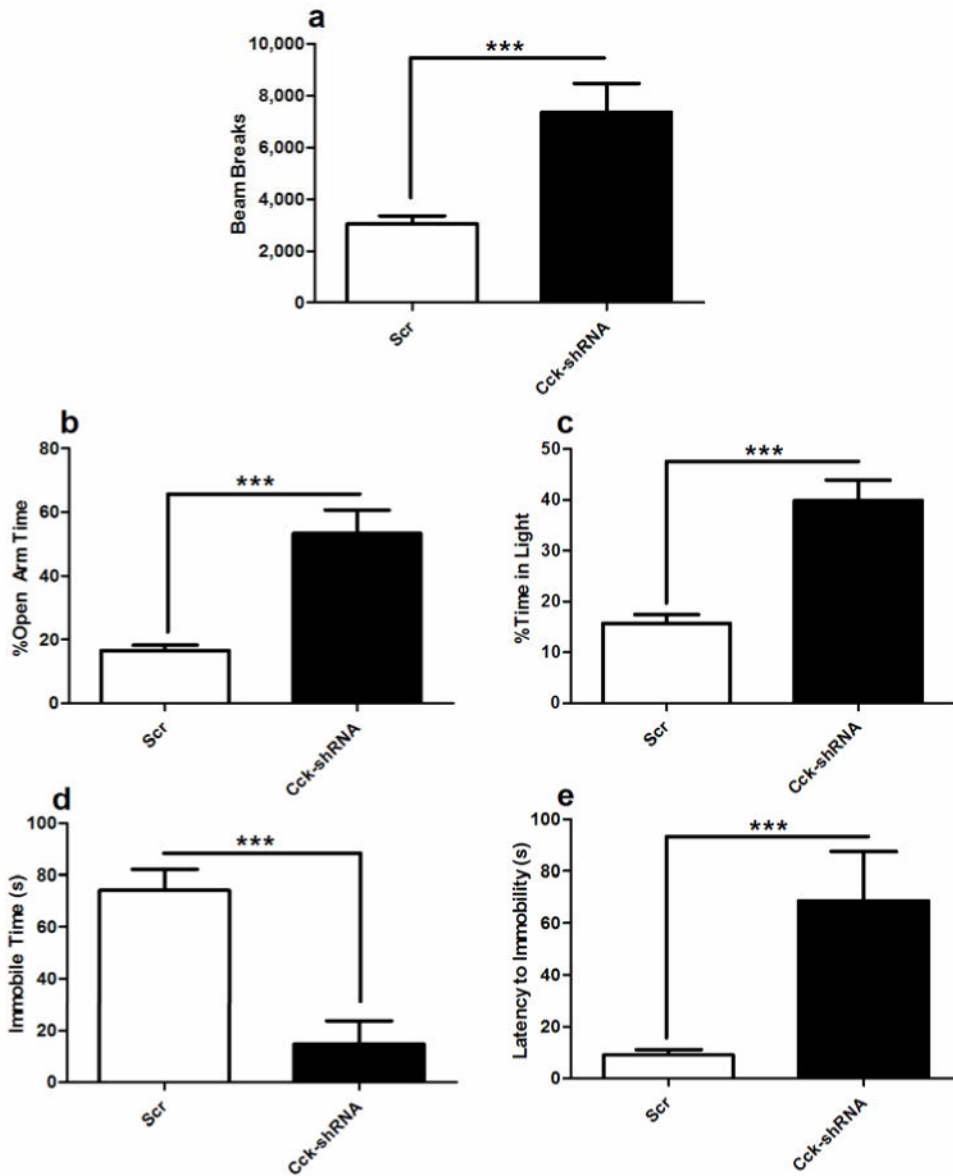
38. Grandjean EM, Aubry JM. Lithium: updated human knowledge using an evidence-based approach: Part I: Clinical efficacy in bipolar disorder. *CNS Drugs*. 2009; 23(3):225–240. [PubMed: 19320531]
39. Malhi GS, Adams D, Berk M. Is lithium in a class of its own? A brief profile of its clinical use. *Aust N Z J Psychiatry*. 2009; 43(12):1096–1104. [PubMed: 20001408]
40. Crump NT, Hazzalin CA, Bowers EM, Alani RM, Cole PA, Mahadevan LC. Dynamic acetylation of all lysine-4 trimethylated histone H3 is evolutionarily conserved and mediated by p300/CBP. *Proc Natl Acad Sci U S A*. 2011; 108(19):7814–7819. [PubMed: 21518915]
41. Guillemette B, Drogaris P, Lin HH, Armstrong H, Hiragami-Hamada K, Imhof A, et al. H3 lysine 4 is acetylated at active gene promoters and is regulated by H3 lysine 4 methylation. *PLoS Genet*. 2011; 7(3):e1001354. [PubMed: 21483810]
42. Martin C, Zhang Y. The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol*. 2005; 6(11):838–849. [PubMed: 16261189]
43. Nightingale KP, Gendreizig S, White DA, Bradbury C, Hollfelder F, Turner BM. Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. *J Biol Chem*. 2007; 282(7):4408–4416. [PubMed: 17166833]
44. Doi M, Hirayama J, Sassone-Corsi P. Circadian regulator CLOCK is a histone acetyltransferase. *Cell*. 2006; 125(3):497–508. [PubMed: 16678094]
45. Zhao WN, Malinin N, Yang FC, Staknis D, Gekakis N, Maier B, et al. CIPC is a mammalian circadian clock protein without invertebrate homologues. *Nat Cell Biol*. 2007; 9(3):268–275. [PubMed: 17310242]
46. Moretti M, Valvassori SS, Varela RB, Ferreira CL, Rochi N, Benedet J, et al. Behavioral and neurochemical effects of sodium butyrate in an animal model of mania. *Behav Pharmacol*. 2011; 22(8):766–772. [PubMed: 21989497]



**Figure 1. *Cck* is a CLOCK target gene and is regulated by the E-box element**  
**(a)** Diagram of the *Cck* promoter. The region containing the proximal promoter, including the E-Box, was amplified by quantitative qPCR after 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* 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* 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 5 $\mu$ g of CLOCK 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). (d) Relative enrichment of MLL1 at the *Cck* promoter in *Clock*<sup>-19</sup> 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*<sup>-19</sup> mice ( $t_8=2.827$ ,  $p=0.0223$ ); (n= 5 per group). (e) Relative enrichment of H3K4me3 at the *Cck* promoter in *Clock*<sup>-19</sup> mice and WT littermates was assessed by performing ChIP assays with an H3K4me3-specific antibody. A significant decrease in MLL1 at the *Cck* promoter was observed in *Clock*<sup>-19</sup> mice ( $t_8=2.377$ ,  $p=0.0415$ ); (n= 5 per group). In all panels error bars show S.E.M.





**Figure 2. Knockdown of *Cck* in the VTA of wild type animals results in a manic-like phenotype** (a) Locomotor activity of AAV-Cck-shRNA and AAV-Scr injected C57BL/6J animals was assessed for two hours, 3 weeks after stereotaxic injection. AAV-Cck-shRNA injected animals are hyperactive when compared to AAV-Scr controls ( $t_{18}= 3.756$ ,  $p < 0.01$ ). (b–c) Anxiety-related behavior was assessed in AAV-Cck-shRNA and AAV-Scr injected animals using (b) the elevated plus maze (EPM) and (c) dark/light box. AAV-Cck-shRNA injected animals are significantly less anxious than AAV-Scr controls as seen by (b) an increase in open arm time on the EPM ( $t_{16}=4.90$ ,  $p < 0.001$ ) 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 mice 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

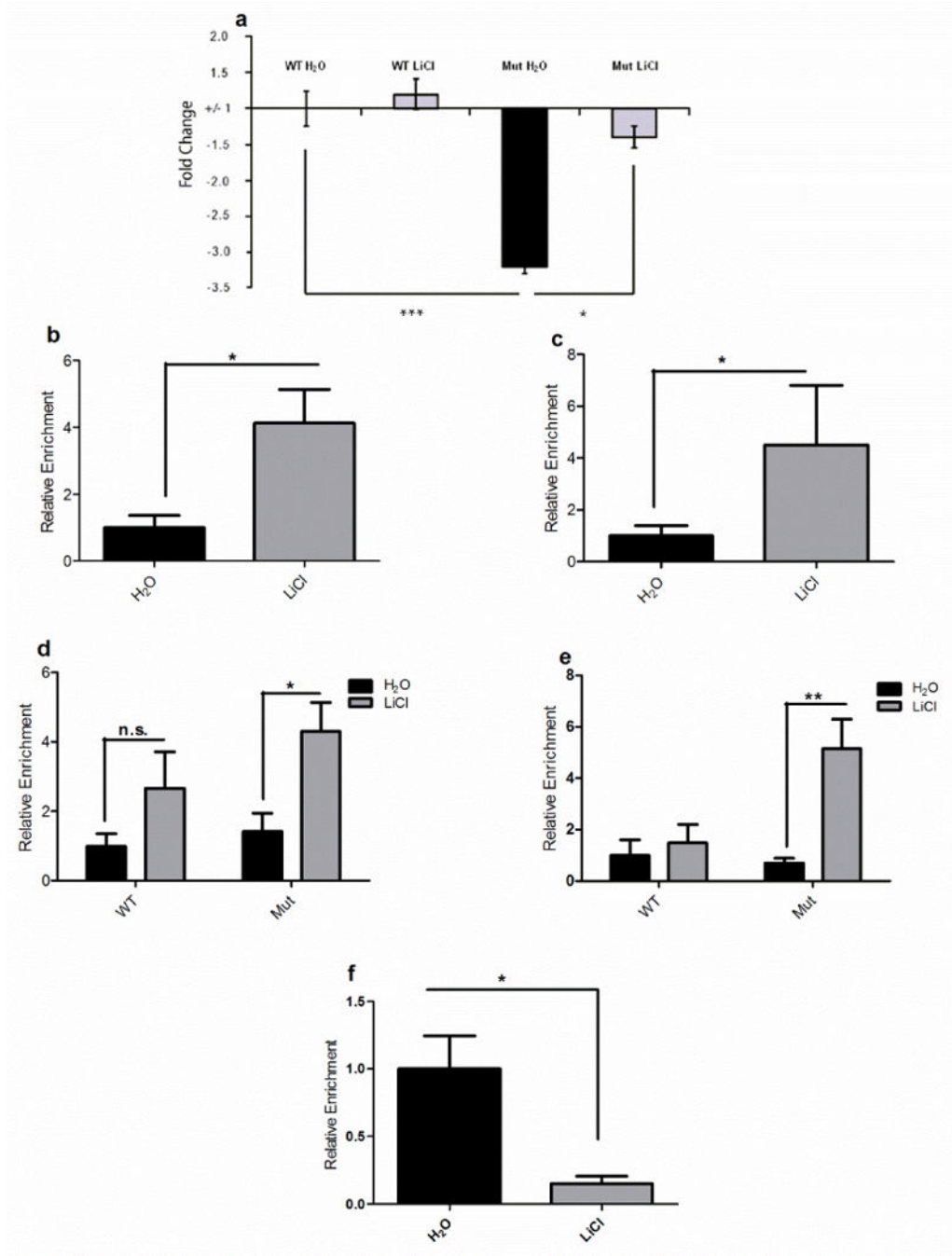
increased latency to first bout of immobility ( $t_{14}=3.126$ ,  $p < 0.01$ ); ( $n=15-20$  per group). In all panels error bars show S.E.M.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 3. Effect of the *Clock* 19 mutation and lithium treatment on *Cck* expression and H3k4me3 and MLL1 binding at the *Cck* promoter**  
**(a)** Relative mRNA levels of *Cck* in *Clock* 19 mice and WT littermates receiving 10 days of water or lithium treatment (600 mg/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* 19 mice compared to WT animals (main effect of genotype  $F_{1,20}=16.99$ ;  $p < 0.001$ ). Bonferroni post hoc tests revealed that lithium treatment caused a significant increase in *Cck* expression in *Clock* 19 mice relative to water alone, restoring it to near WT

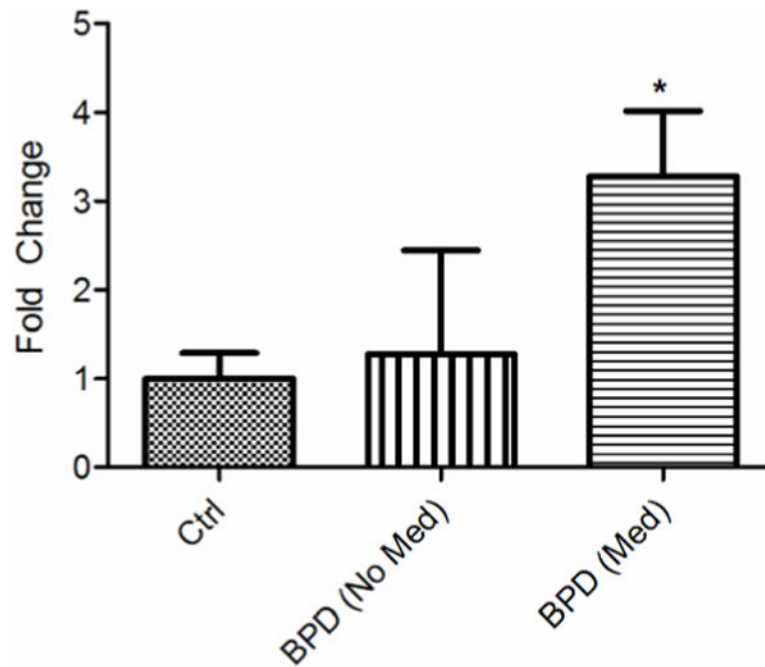
Author Manuscript

Author Manuscript

Author Manuscript

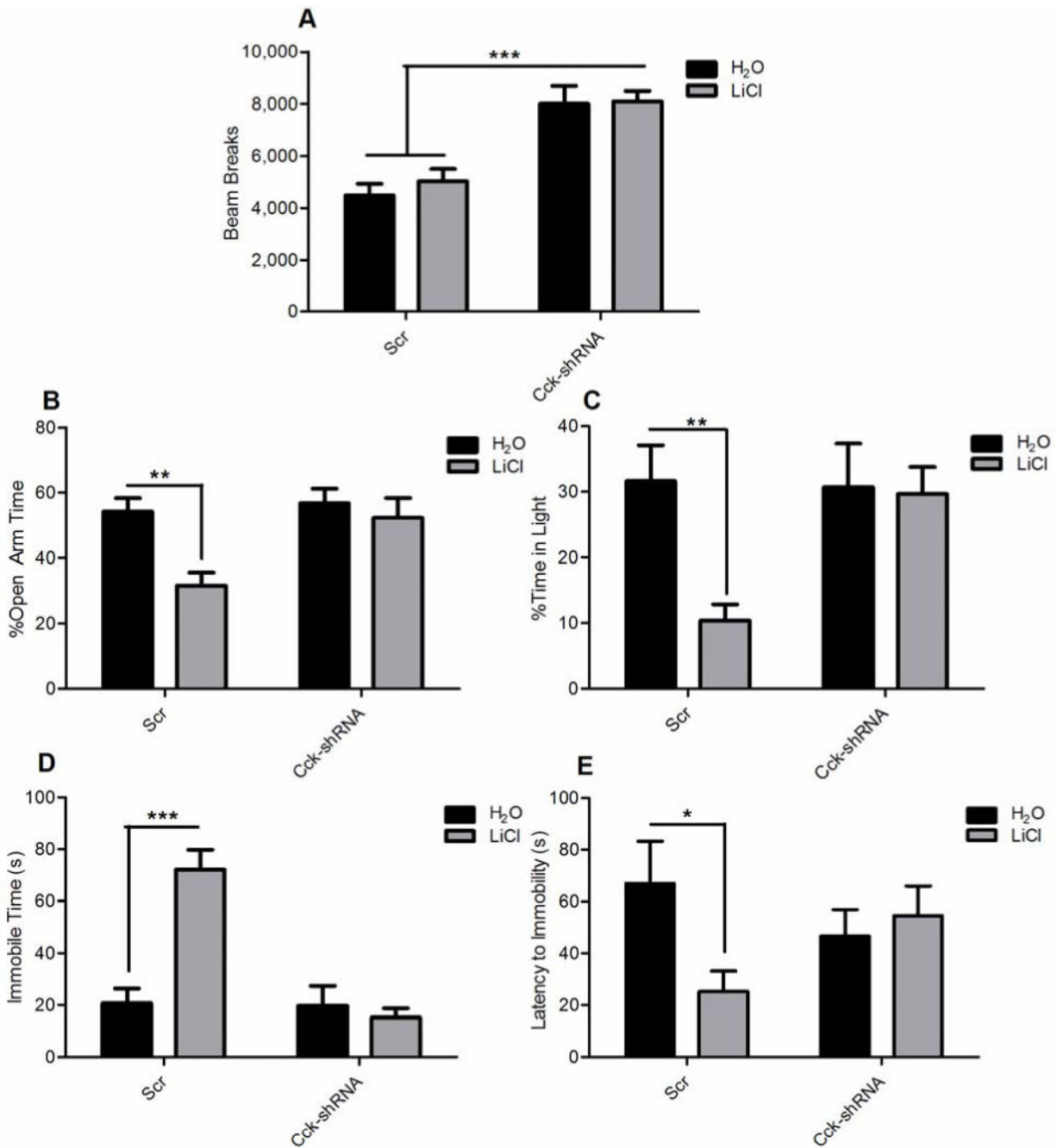
Author Manuscript

levels ( $t=2.600$ ,  $p < 0.05$ ). Lithium treatment had no detectable effect on WT *Cck* expression. In all panels error bars show S.E.M. **(b)** Relative levels of histone H3K4me3 at the *Cck* promoter in *Clock*<sup>-19</sup> mice following lithium treatment were assessed by performing ChIP assays with a H3K4me3 specific antibody. Lithium treatment caused a significant increase in levels like H3K4me3 at the *Cck* promoter ( $t_9 = 2.690$ ,  $p = 0.0248$ ); ( $n=5-6$  per group). **(c)** Relative enrichment of MLL1 at the *Cck* promoter in *Clock*<sup>-19</sup> mice following lithium treatment was assessed by performing ChIP assays with an MLL1-specific antibody. There was no significant change in MLL1 levels in lithium-treated *Clock*<sup>-19</sup> mice ( $t_8=1.865$ ,  $p = 0.0992$ ); ( $n=5-6$  per group). **(d)** Relative levels of acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) at the *Cck* promoter in *Clock*<sup>-19</sup> (Mut) mice and WT littermates following lithium (LiCl) treatment were assessed by performing ChIP assays with a AcH3 and AcH4 specific antibodies. Analysis by two-way ANOVA revealed a main effect of lithium treatment on AcH3 ( $d, F_{1,20}=9.4$ ,  $p=0.0061$ ) and AcH4 ( $e, F_{1,17}=11.32$ ,  $p=0.0037$ ) 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*<sup>-19</sup> mice following lithium treatment, while lithium had no detectable effect on WT animals; ( $n= 5-6$  per group). **(f)** Relative enrichment of CLOCK at the *Cck* promoter in *Clock*<sup>-19</sup> mice following lithium treatment was assessed by performing ChIP assays with a CLOCK-specific antibody. A significant decrease in CLOCK binding at the *Cck* promoter was observed in lithium-treated *Clock*<sup>-19</sup> mice ( $t_9=3.137$ ,  $p < 0.05$ ); ( $n=5-6$  per group). In all panels error bars show S.E.M.



**Figure 4. Regulation of *Cck* levels in the VTA of BPD patients by mood stabilizers**

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).  $n$  all panels error bars show S.E.M.



**Figure 5. Increased *Cck* in the VTA is required for lithium’s therapeutic actions in the *Clock* 19 mice**

(a) Locomotor activity was measured in AAV-Cck-shRNA or AAV-Scr injected *Clock* 19 mice for two hours following 10 days of lithium 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 hoc tests revealed that there was no 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* 19 mice following lithium treatment using the EPM (b) and dark/light box (c). Analysis by two-way ANOVA followed by Bonferroni post hoc



tests revealed that lithium treatment caused 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* 19 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). In all panels error bars show S.E.M.