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The Role of *Clock* in Ethanol-Related Behaviors

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Mice with a mutation in the *Clock* gene (*Clock*Δ19) exhibit increased preference for stimulant rewards and sucrose. They also have an increase in dopaminergic activity in the ventral tegmental area (VTA) and a general increase in glutamatergic tone that might underlie these behaviors. However, it is unclear if their phenotype would extend to a very different class of drug (ethanol), and if so, whether these systems might be involved in their response. Continuous access voluntary ethanol intake was evaluated in *Clock*Δ19 mutants and wild-type (WT) mice. We found that *Clock*Δ19 mice exhibited significantly increased ethanol intake in a two-bottle choice paradigm. Interestingly, this effect was more robust in female mice. Moreover, chronic ethanol experience resulted in a long-lasting decrease in VTA *Clock* expression. To determine the importance of VTA *Clock* expression in ethanol intake, we knocked down *Clock* expression in the VTA of WT mice via RNA interference. We found that reducing *Clock* expression in the VTA resulted in significantly increased ethanol intake similar to the *Clock*Δ19 mice. Interestingly, we also discovered that *Clock*Δ19 mice exhibit significantly augmented responses to the sedative effects of ethanol and ketamine, but not pentobarbital. However, their drinking behavior was not affected by acamprosate, an FDA-approved drug for the treatment of alcoholism, suggesting that their increased glutamatergic tone might underlie the increased sensitivity to the sedative/hypnotic properties of ethanol but not the rewarding properties of ethanol. Taken together, we have identified a significant role for *Clock* in the VTA as a negative regulator of ethanol intake and implicate the VTA dopamine system in this response. *Neuropsychopharmacology* (2013) **38,** 2393–2400; doi:10.1038/npp.2013.138; published online 17 July 2013

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INTRODUCTION

The majority of living organisms display daily cycles in behavior and physiology that enable them to adapt to their environment and react to a variety of stimuli known as zeitgebers or 'time-givers' (eg, light, food, etc). In mammals, the central circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus, is entrained by light and controls activity rhythms. The SCN coordinates other oscillators in the brain and in peripheral organs (Reppert and Weaver, 2002). Thus, circadian clocks are present throughout the body and regulate numerous metabolic and behavioral rhythms. In mammals, two core components of the molecular clock are the circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1) proteins. CLOCK and BMAL1 are transcription factors that heterodimerize and promote transcription of the *Period* genes (*Per1*, *Per2*, and *Per3*), the *Cryptochrome* genes (*Cry1* and *Cry2*), as well as many other genes by binding the E-box elements in their promoters (Reppert and Weaver, 2001; Takahashi *et al*, 2008). Circadian genes and proteins are widely expressed throughout the brain. Further, there exist SCN-independent pacemakers that can entrain to nonphotic stimuli, like food and drugs (Iijima *et al*, 2002; Stephan, 1984). In addition, drugs of abuse have been suggested to act as zeitgebers, as they can entrain locomotor activity rhythms when given daily (Kosubud *et al*, 1998, 2007). Physiological, behavioral, and molecular rhythms are all affected by rewarding stimuli, including drugs of abuse and food. Rodent studies have shown that prolonged alcohol treatments can disrupt the circadian pattern of a variety of hormonal and behavioral rhythms (Kakihana and Moore, 1976; Kosobud *et al*, 2007, Madeira *et al*, 1997; Rajakrishnan *et al*, 1999; Rosenwasser *et al*, 2005; Spanagel *et al*, 2005a). However, the molecular mechanisms of these disruptions are yet to be determined.

Genetic animal models have also revealed that circadian genes are important regulators of behavioral responses to drugs of abuse. The first studies that revealed this relationship were carried out in *Drosophila melanogaster*, showing that flies bearing a mutation in the circadian genes *Clock*, *Per*, *Cycle*, or *Doubletime* all fail to sensitize to cocaine (Andretic *et al*, 1999). Following these studies, Abarca *et al* (2002) found that *mPer1*^{Brdm}-null mutant mice also fail to sensitize to cocaine and exhibit decreased cocaine conditioned place preference (CPP), while

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mPer2^{Brdm}-null mutants exhibit hypersensitization to cocaine and strong cocaine CPP. Furthermore, studies have shown that *Per2*^{Brdm} mutants are hypersensitive to ethanol; they exhibit increased ethanol preference and consumption, increased sedation, and decreased hypothermia (Liu *et al*, 2005; Perreau-Lenz *et al*, 2009).

Alcoholism in human populations is associated with disruptions in circadian rhythms, which can persist during abstinence and increase risk for relapse (Brower, 2001; Fonzi *et al*, 1994; Kuhlwein *et al*, 2003; Landolt and Gillin, 2001; Sano *et al*, 1993). In addition, studies have reported in rodents that chronic consumption of high levels of ethanol can alter *rPer2* gene expression in the SCN and activity rhythms (Chen *et al*, 2004; Seggio *et al*, 2009). Furthermore, variations (SNPs or mutations) in the *Clock* gene and the *Per2* gene associate with increased alcohol consumption in humans (Spanagel *et al*, 2005b; Sjoholm *et al*, 2010). The role of *Clock* in the reinforcing and motivational aspects of ethanol intake remains unknown. McClung *et al* (2005) and Ozburn *et al* (2012) identified an important role for *Clock* in the regulation of cocaine reward and self-administration. Mice bearing a dominant-negative mutation in *Clock* (*Clock* Δ19 mice) exhibit increased cocaine sensitivity and preference (King *et al*, 1997; McClung *et al*, 2005; Ozburn *et al*, 2012; Vitaterna *et al*, 1994). Furthermore, these mice exhibit increased locomotor activity, reduced anxiety- and depression-like behavior, increased intracranial self-stimulation (ICSS) at a lower threshold, and increased dopaminergic cell activity in the ventral tegmental area (VTA) (McClung *et al*, 2005; Roybal *et al*, 2007). Many of these behavioral phenotypes are rescued by expressing functional CLOCK in the VTA of *Clock*Δ19 mutants or are recapitulated by reducing *Clock* expression in the VTA of wild-type (WT) mice via RNA interference (RNAi; Mukherjee *et al*, 2010; Roybal *et al* 2007). What remains unknown is if *Clock* in the VTA is important for the response to only stimulant drugs, or if it also has a role in ethanol consumption and ethanol-related behaviors. The aim of this study was to determine if *Clock* is a mediator of ethanol intake. Further examination of the role of circadian genes in ethanol-related behaviors has important translational significance for treatment, as mutations in circadian genes might increase the vulnerability for alcoholism.

MATERIALS AND METHODS

Mice

*Clock*Δ19 mutant mice were created by *N*-ethyl-*N*-nitrosourea mutagenesis, resulting in a dominant-negative CLOCK protein (King *et al*, 1997; Vitaterna *et al*, 1994). The *Clock*Δ19 mutation is maintained on a BALB/c background (>10 generations of backcrossing) by heterozygous breeding and genotyping was carried out using PCR (as reported in Ozburn *et al*, 2012). Female *Clock* mutant (*Clock*Δ19/*Clock*Δ19) and WT littermate controls were used in all experiments. In addition, as described in Supplementary Materials, continuous access two-bottle choice experiments were performed to determine ethanol or quinine preference in male mice. Blizard *et al* (2004) reported female BALB/c mice drink 10% ethanol with a higher preference than male mice (with females exhibiting an estimated preference score of 23% compared with 17% in males). While both male and female *Clock*Δ19 mice exhibit a similar behavioral phenotype, behavioral changes are more robust in female mice in their behavioral responses in measures of activity and anxiety (Easton *et al*, 2003). Mice were 8–12 weeks old at the beginning of experiments and were group housed on a 12 h light/12 h dark cycle with food and water *ad libitum*, unless otherwise specified. All experiments were in compliance with protocols approved by the IACUC at University of Texas Southwestern Medical Center and the University of Pittsburgh.

Continuous Access Two-bottle Choice

As described in Ozburn *et al* (2010), mice were habituated to individual housing and sipper bottles for 1 week before the start of the experiment. Mice were offered water and 3% ethanol (v/v in tap water) for 2 days. After 3% ethanol, escalating concentrations (up to 21%) were offered *vs* water, 2 days each. Fluid intake was measured daily. Mice were weighed every 4 days. Ethanol preference (ml ethanol solution consumed/ml total fluid consumed), ethanol consumption (g pure ethanol/kg body weight per day), and total fluid consumption were measured ($n = 9$–11 per genotype).

Loss of Righting Reflex

Mice were injected with ethanol (3.6 g/kg) at zeitgeber 5 (ZT5; $n = 7$–8 per genotype) or ZT11 ($n = 6$–7 per genotype), pentobarbital (100 mg/kg; $n = 9$ per genotype) at ZT5, or ketamine (50 mg/kg; $n = 9$ per genotype) at ZT7-8, and when they became ataxic, they were placed in the supine position until they were able to right themselves three times within 60 s. Data for three mice were excluded from the ZT11 ethanol trial because of misplaced injection.

Gene Expression

As it has been shown that recently abstinent alcoholics have reduced *Clock* expression, we measured *Clock* expression in reward-related brain regions after the continuous access two-bottle choice and 1 week of abstinence (Huang *et al*, 2010). Mice were killed at two time points over the circadian cycle corresponding to ZT4 and ZT16. Our group has observed peaks and troughs in *Clock* gene expression in the nucleus accumbens (NAc) and VTA at approximately ZT4-8 and ZT16-20. Brains were removed and frozen on powdered dry ice. NAc and VTA punches were taken on a freezing stage from 300-μM sections taken on a cryostat. RNA isolation and cDNA synthesis were carried out as described previously (McClung *et al*, 2005). Real-time PCR was performed in duplicate using Power SYBR Green PCR Master Mix and the ABI 7900 Real-Time PCR system (Applied Biosystems, Carlsbad, CA) with primers specific for *18s* and *Clock* (*18s* forward: 5′-ACCGCAGCTAGGAATA ATGGA-3′, *18s* reverse: 5′-GCCTCAGTTCCGAAAACCA-3′; *Clock* forward: 5′-CAGAACAGTACCCAGAGTGCT-3′, *Clock* reverse: 5′-CACCACCTGACCCATAAGCAT-3′) (Mukherjee *et al*, 2010). Relative expression levels were determined by normalizing the CTs (cycle thresholds) for *Clock* to corresponding *18s* CTs and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method ($n = 5$–10 mice per ZT per treatment).

AAV Purification

Construction, production, purification, and validation of the *Clock* and scramble shRNAs are described in Mukherjee *et al* (2010). Viral production and shRNA design were carried out using a helper-free triple transfection method in human embryonic kidney 293 cells (American Type Culture Collection, Manassas, VA). The AAV *Clock* and scramble shRNA vectors also express GFP. Sequences and other details are in Mukherjee *et al* (2010).

Stereotaxic Injection of AAV-shRNA into VTA

Stereotaxic surgery was performed as described in Mukherjee *et al* (2010). WT mice (littermates to the *Clock*Δ19 mutants) were anesthetized with a mixture of ketamine (100 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°, A/P −3.2 mm, M/L +1.0, D/V −4.6) of 1 μl AAV-*Clock* shRNA or AAV-scramble shRNA (1×10^{12} infectious particles per ml) were performed using a 33-G Hamilton syringe (Hamilton, Reno, NV). The virus was injected (0.1 μl/min) and the needle was kept in place for 5 min before it was withdrawn. Mice recovered for 3 weeks before behavioral testing ($n = 12$–13 per treatment).

Immunohistochemistry and Validations of Stereotaxic Injection Placement/AAV Infection

As described in Mukherjee *et al* (2010), mice were deeply anesthetized (175 mg/kg sodium pentobarbital) and perfused intracardially with 0.01 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were removed, postfixed in 4% paraformaldehyde overnight at 4 °C, placed in 30% glycerol in PBS for 24 h, and then placed in PBS with 0.01% sodium azide. VTA containing sections (30 μm) were collected using a freezing 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 Alexa 488 and 1 : 400 anti-rabbit rhodamine) were purchased from Invitrogen (Carlsbad, CA). Immunostained sections were mounted using Vectashield with DAPI (Vector Labs, Burlingame, CA) and observed with an epifluorescence microscope to identify the location of viral injection and levels of infection. As described in Mukherjee *et al* (2010), VTA cells that displayed more than 50% of GFP colocalization with TH antibody staining were included for behavioral analysis (two mice were eliminated from analysis based on this criteria; thus, the final number of mice included in the analysis is $n = 12$–13 per treatment).

Statistical Analysis

All data are expressed as mean ± SEM. Two-bottle choice data, ethanol-induced loss of righting reflex (LORR), and gene expression data were analyzed by two-way analysis of variance (ANOVA). Repeated measures were applied where appropriate. Significance for data for the ketamine- and pentobarbital-induced LORR experiments were determined by Student's *t*-test. In all experiments, $p < 0.05$ is considered significant. The presence of outliers was identified (defined as greater than or less that two times the standard deviation of the mean) for ethanol two-bottle choice (for all experiments combined eight mice showed side preference, only drinking from one side regardless of solution in bottle) and LORR testing (three mice did not lose their righting reflex) and these data were removed before statistical analysis.

RESULTS

*Clock*Δ19 Exhibit Increased Ethanol Preference and Consumption

To determine if functional CLOCK is important for voluntary ethanol drinking, we measured ethanol preference and consumption in *Clock*Δ19 and WT littermates using the continuous access two-bottle choice paradigm. Ethanol intake in this paradigm positively correlates with operant ethanol self-administration (Greene and Grahame, 2007). *Clock*Δ19 female mice exhibited significantly increased ethanol consumption and preference, without any effect on total fluid consumption (Figures 1a–c; ethanol consumption: genotype × concentration interaction—$F(6,108) = 3.69$, $p < 0.01$, main effect of genotype—$F(1,18) = 5.17$, $p < 0.05$, main effect of concentration—$F(6,108) = 58.18$, $p < 0.0001$; ethanol preference: main effect of genotype—$F(1,18) = 7.11$, $p < 0.05$, main effect of concentration—$F(6,108) = 7.80$, $p < 0.0001$). Ethanol preference and consumption data for

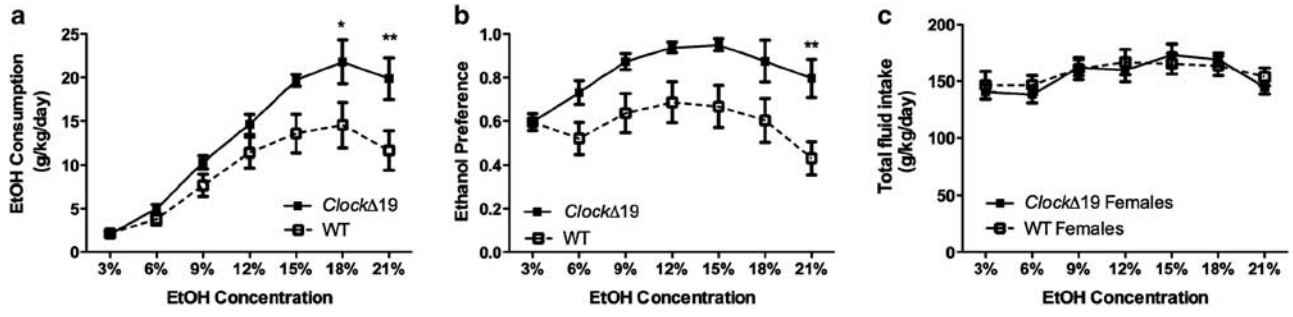

Figure 1 *Clock*Δ19 mice exhibit significantly increased ethanol (EtOH) intake. (a) Ethanol consumption, (b) ethanol preference, and (c) total fluid intake. *$p < 0.05$; **$p < 0.01$. WT, wild type.

both genotypes displayed a typical inverted U shape. Bonferroni *post hoc* analysis revealed that *Clock*Δ19 mice consumed significantly more ethanol and showed a higher preference for high ethanol concentrations. Male Clock mutants exhibited a strong trend toward significantly increased ethanol preference (Supplementary Figure 1).

As preference for ethanol can be influenced by taste, we examined preference for the bitter tastant, quinine, in male and female WT and *Clock*Δ19 mice. We found that both male and female WT and *Clock*Δ19 mice exhibit a similar taste avoidance profile for quinine solutions (Supplementary Figure 2). We have previously reported a modest increase in sucrose preference for *Clock*Δ19 mice (Roybal *et al,* 2007).

*Clock*Δ19 Mice Exhibit Augmented Responses to the Sedative Effects of Ethanol and Ketamine, but not Pentobarbital

Perreau-Lenz *et al* (2009) reported a diurnal variation in sensitivity to the sedative effects of ethanol, with greatest duration of LORR at ZT11 and shortest duration at ZT5. In this study, we found that latency to ethanol-induced LORR was similar for both genotypes; however. the duration of LORR produced by 3.6 g/kg ethanol was significantly greater for *Clock*Δ19 mice at ZT5 and ZT11 (Figures 2a and b; LORR duration: genotype × ZT interaction—$F(1,24) = 7.92$, $p < 0.01$, main effect of genotype—$F(1,24) = 234.6$, $p < 0.0001$). Diurnal variation in sensitivity to ethanol's sedative effects is absent in *Clock*Δ19 mice. It is possible that *Clock*Δ19 mice may be more sensitive/responsive to other sedative drugs; thus, we tested whether the LORR effect was specific to ethanol. As ethanol has been shown to act directly on GABAa and NMDA receptors, we tested pentobarbital- and ketamine-induced LORR (Harris *et al*, 2008). *Clock*Δ19 mice exhibited a significantly shorter latency to ketamine-induced LORR and greater duration of LORR ($p < 0.05$) than WT mice (Figures 2c and d). However, both genotypes showed a similar latency to and duration of LORR to pentobarbital (Figures 2e and f). Taken together, these results suggest that the increased sensitivity to the sedative/hypnotic effects of ethanol seen in *Clock*Δ19 mice may be because of a hyperglutamatergic system and are likely not due to differences in GABAergic transmission. Gupta *et al* (2008) reported that glutamatergic signaling has a role in binge-like ethanol drinking in the drinking in the dark (DID) paradigm, where they reported that mice pretreated with acamprosate or MPEP (an mGluR5 antagonist) exhibited decreased DID. Perhaps, the altered glutamatergic tone indirectly measured by ketamine LORR and directly measured and described by Beaulé *et al* (2009) in *Clock*Δ19 mice would lead to increased binge-like ethanol drinking in the DID paradigm. We found that *Clock*Δ19 and WT mice exhibit comparable ethanol intake in the DID paradigm and respond to acamprosate similarly (data not shown). This suggests that the increased sensitivity to ketamine seen in *Clock*Δ19 mutants (and possible altered glutamatergic tone) may not have an important role in their binge-like ethanol consumption. Furthermore, we found no other obvious predictors of their increased ethanol intake in a behavioral battery (including ethanol-induced taste aversion and acute functional tolerance) (Supplementary Figure 3). *Clock*Δ19 and WT mice exhibit similar behavioral and metabolic responses to ethanol.

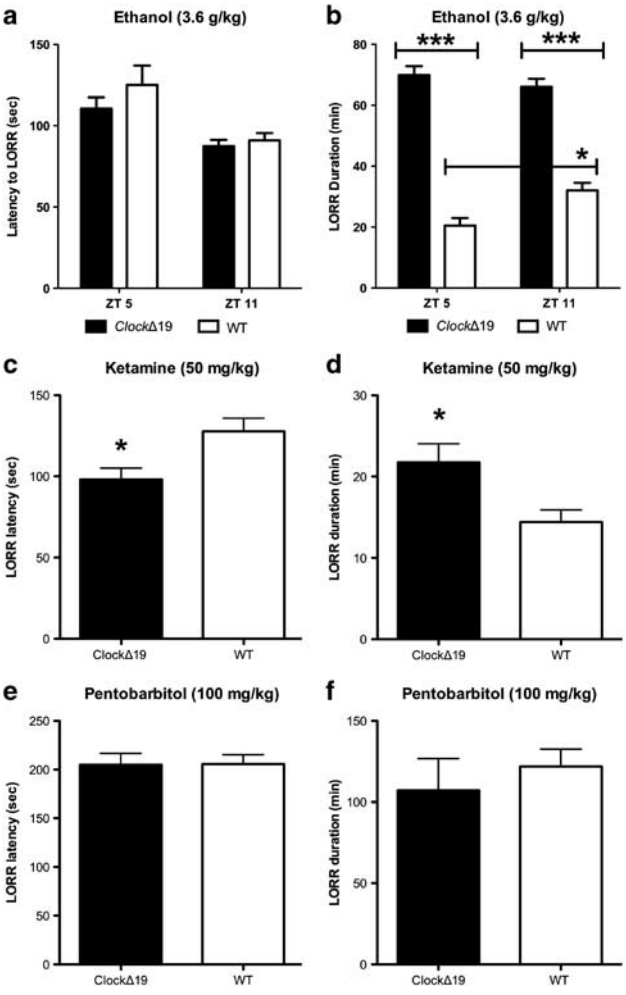

Figure 2 *Clock*Δ19 mice exhibit augmented responses to the sedative effects of ethanol and ketamine. Latency (a) and duration (b) to ethanol-induced loss of righting reflex (LORR) at ZT5 and ZT11. Latency (c) and duration (d) to ketamine-induced LORR. Latency (e) and duration (f) to pentobarbitol-induced LORR. *$p < 0.05$; ***$p < 0.001$. WT, wild type; ZT, zeitgeber time.

Decreased *Clock* Expression in Reward-Related Brain Regions after Chronic Ethanol Intake and Abstinence

Decreased plasma *Clock* expression has been reported for recently abstinent alcoholics (Huang *et al*, 2010). We hypothesized that this phenomenon would extend to reward-related brain regions, specifically the NAc and VTA. To determine if *Clock* expression in the NAc and VTA was modulated by ethanol consumption, WT mice voluntarily consumed ethanol in a choice paradigm for 2 weeks, were subjected to 1 week of abstinence, and were then killed at ZT4 or ZT16. *Clock* mRNA expression in NAc and VTA was quantified via qPCR. *Clock* expression in the NAc was significantly lower in ethanol-experienced mice in a ZT-dependent manner (Figure 3a, main effect of ZT—$F(1,24) = 13.55$, $p < 0.05$; main effect of treatment—$F(1,24) = 234.6$, $p < 0.05$). *Clock* expression in VTA was significantly decreased in ethanol-experienced mice at both times

of day tested (Figure 3b, main effect of treatment—$F(1,26) = 6.25$, $p < 0.05$). As chronic ethanol intake and abstinence resulted in decreased VTA *Clock* expression at both times of day tested, similar to what is expected when RNAi is invoked, we focused on the VTA for the next experiment.

Clock Knockdown in the VTA Leads to Increased Ethanol Preference and Consumption in WT Mice

To test if decreased VTA *Clock* expression was sufficient to produce increased ethanol consumption and preference, we used viral-mediated gene transfer of an shRNA to knock down *Clock* expression in the VTA in WT mice and measured ethanol preference and consumption using the continuous access two-bottle choice paradigm. Knocking down *Clock* expression in the VTA led to significantly increased ethanol consumption and preference, without affecting total fluid intake (Figures 4a–c; ethanol consumption: main effect of treatment—$F(1,161) = 11.30$, $p < 0.001$, main effect of concentration—$F(6,161) = 14.40$, $p < 0.0001$; ethanol preference: main effect of treatment—$F(1,161) = 17.02$, $p < 0.0001$, main effect of concentration—$F(6,161) = 3.85$, $p < 0.01$). Representative image of VTA-specific targeting of AAV *Clock* shRNA is shown in Figure 4d.

DISCUSSION

Results of this study establish that CLOCK is involved in regulating ethanol intake. *Clock*Δ19 mice exhibit significantly increased ethanol intake, with female mice exhibiting a more robust phenotype. Moreover, chronic ethanol intake and abstinence results in a long-lasting decrease in NAc and VTA *Clock* expression in WT mice. The most compelling evidence showing VTA *Clock* is a negative regulator of ethanol intake is shown in the RNAi experiment, where we found that shRNA-mediated reduction of *Clock* results in increased ethanol intake similar to what is observed in the *Clock*Δ19 mice. These findings demonstrate the involvement of the circadian transcription factor, CLOCK, in regulating ethanol intake.

A growing body of evidence implicates glutamatergic systems in drug-related behaviors and drug-induced plasticity. Pharmacological approaches targeting glutamatergic systems for the treatment of alcoholism have proven utility. Acamprosate is an FDA-approved treatment for reducing craving and relapse in alcoholics (Olive *et al*, 2012; Yahn *et al*, 2013). Although the exact mechanism of action has yet to be determined, studies have shown that acamprosate can act as an NMDA modulator and is thought to restore balance to perturbations in excitatory and inhibitory neurotransmission (Olive *et al*, 2012). Recent studies have revealed evidence of altered glutamatergic systems in *Clock*Δ19 mice that may contribute to their drug-preferring phenotype (Beaulé *et al*, 2009; Dziraza *et al*, 2010). Beaulé *et al* (2009) showed that functional CLOCK and Per2 proteins are important for regulating glutamate levels. *Clock*Δ19 mice have reduced mRNA and protein levels of the glial excitatory amino-acid transporter, EAAT1, as well as reduced glutamate uptake. Further, Spanagel *et al* (2005a, b) reported that mutation of another circadian gene, *Per2*, also results in reduced EAAT1 expression and increased alcohol intake. Thus, it is possible that the decreased glutamate uptake and increased NMDA receptor levels result in a hyperglutamatergic tone and this could contribute to the observed increase in neuronal excitability and increased sensitivity to ethanol (and ketamine) observed in *Clock*Δ19 mice. However, we found that acamprosate fails to reduce differentially ethanol intake (in a binge-drinking paradigm) in the *Clock*Δ19 mice, suggesting that the altered glutamatergic tone is not responsible for the increase in alcohol intake. Further, Brager *et al* (2011) reported that acamprosate decreases ethanol drinking in a similar manner for WT and Per2 mutants. Nevertheless, the finding that *Clock*Δ19 mice are more sensitive to the sedative/hypnotic effects of ketamine but not pentobarbital suggests that the altered glutamatergic tone in these mice is likely involved in the increased sedative/hypnotic properties of alcohol.

The mesolimbic dopaminergic system has long been implicated in motivational behaviors, including drug-induced behaviors and neuronal plasticity (Nestler, 2005; Schultz, 1998). Drugs of abuse increase levels of the rate-limiting enzyme in dopamine synthesis, *Th* (tyrosine hydroxylase), and augment firing rates of VTA dopamine neurons via direct and indirect mechanisms (Nestler, 2005; Stuber *et al*, 2008). Importantly, CLOCK is involved in regulating dopaminergic transmission. *Clock*Δ19 mice have increased tonic and phasic dopamine transmission, as well as cell volume changes (McClung *et al*, 2005; Coque *et al*, 2011). Augmented DA firing is associated with increased locomotor activity and drug reward, which is also seen in *Clock*Δ19 mice (Adamantidis *et al*, 2011; Marinelli and White, 2000; McClung *et al*, 2005; Ozburn *et al*, 2012). Knocking down *Clock* expression in the VTA recapitulates the increased DA cell firing seen in *Clock*Δ19 mice (Mukherjee *et al*, 2010). In addition, overexpressing an inwardly rectifying potassium channel subunit (Kir2.1) selectively in the VTA of *Clock*Δ19 mice reduces the firing rate of dopamine neurons in *Clock*Δ19 mice and results in a normalization of their locomotor- and anxiety-related behaviors (Coque *et al*, 2011). Taken together, these studies show that abnormalities in dopamine cell firing and associated morphology underlie alterations in locomotor, anxiety-, and drug-related behavior.

The regulation of the VTA dopamine system by CLOCK is likely achieved through its actions as a transcription factor. We have previously reported that several genes in the VTA

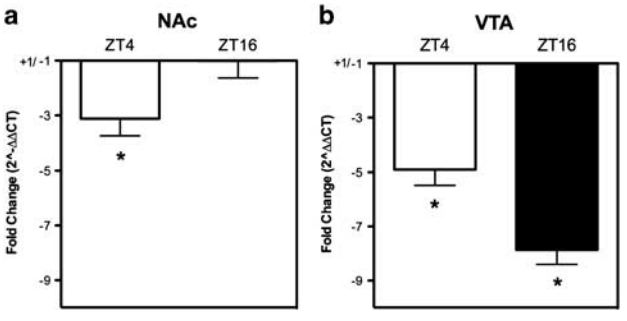

Figure 3 Chronic ethanol intake and abstinence results in significantly decreased nucleus accumbens (NAc) and ventral tegmental area (VTA) *Clock* expression. *Clock* expression in the NAc (a) and VTA (b) at ZT4 and ZT16 was normalized to *18s*, and fold change was calculated using the ΔΔCT method (ΔCTethanol − ΔCTcontrol). *$p < 0.05$. ZT, zeitgeber time.

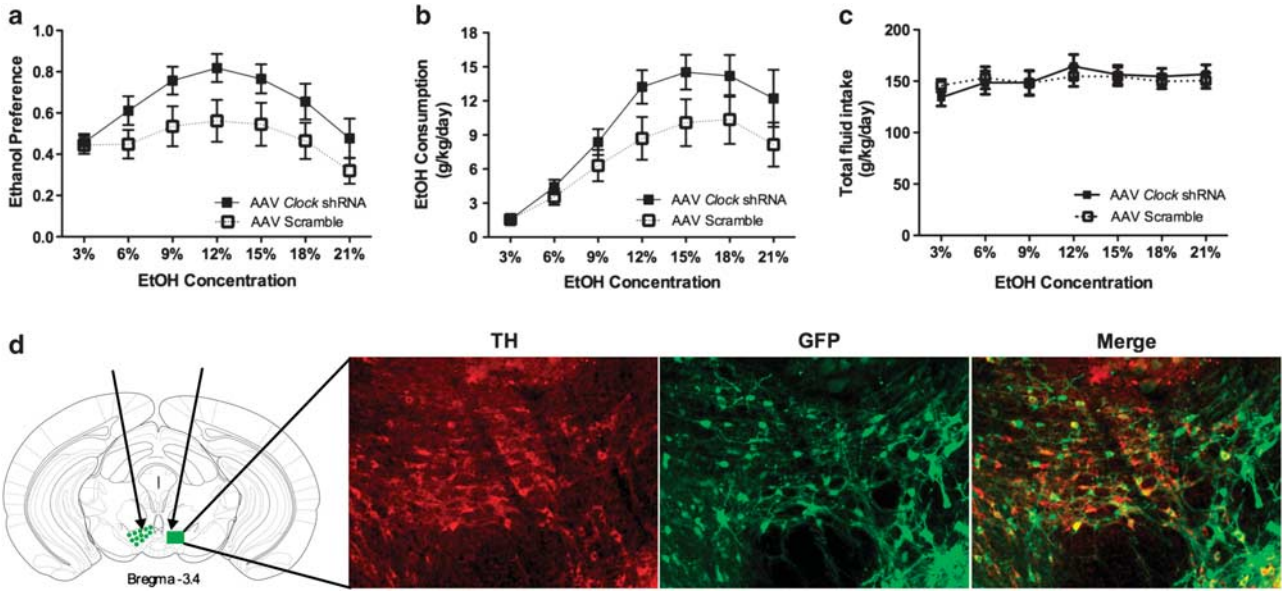

Figure 4 Reducing *Clock* in the ventral tegmental area (VTA) via RNA interference (RNAi) results in significantly increased ethanol intake. (a) Ethanol (EtOH) consumption, (b) EtOH preference, (c) total fluid intake, and (d) diagram of VTA containing coronal section, where green indicates VTA regions targeted by stereotaxic injection (indicated by arrows). Representative photomicrographs showing VTA-specific targeting of adeno-associated virus (AAV) *Clock* short hairpin RNA (shRNA). Brain sections were immunostained with anti-tyrosine hydroxylase (TH) and -green fluorescent protein (GFP) antibodies and 20x images were merged to observe colocalization.

known to control dopaminergic activity are altered in *Clock*Δ19 mice (McClung *et al*, 2005). *Th* expression in the VTA exhibits a circadian pattern, and striatal dopamine levels are increased during the light phase of the light/dark cycle in the *Clock*Δ19 mice (Coque *et al*, 2011). The mechanism underlying CLOCK regulation of *Th* is currently under investigation in our laboratory. In addition to the upregulation of *Th* seen in *Clock*Δ19 mice, we have previously reported downregulation of *Cholecystokinin* (*Cck*). Cck is a peptide neurotransmitter that is colocalized and coreleased with dopamine and acts as a negative modulator of dopaminergic transmission (Ghijsen *et al*, 2001; Hökfelt *et al*, 1980; Lança *et al*, 1998; Voigt *et al*, 1986). Arey *et al* (2009, 2013) have shown that CLOCK binds to an E-box in the *Cck* promoter, and positively regulates *Cck* expression. Furthermore, reducing *Cck* expression in the VTA (via RNAi) recapitulates the behavioral phenotype observed in *Clock*Δ19 mice (decreased anxiety- and depression-like behaviors). Previous studies have shown that increased Cck results in increased anxiety and depression-like behavior, whereas Cck receptor blockade is anxiolytic and antidepressant (Rotzinger *et al*, 2010). The downregulation of Cck in the *Clock*Δ19 mice is particularly interesting in the context of this study as Cck receptors have been implicated in alcohol-related behaviors in both rodent and human studies (Crespi, 1998; Miyasaka *et al*, 2004, 2005).

Chronic alcohol treatment leads to lasting changes in *Clock* gene expression, which likely contributes to the pervasive disruptions in rhythms and sleep in alcoholics. Although we did not measure *Clock* expression in the SCN, others have shown that the SCN is responsive to ethanol (Chen *et al*, 2004; Prosser and Glass, 2009; Seggio *et al*, 2009). Studies in different species have illustrated that circadian clock neurons are important for alcohol tolerance and that alcohol can modulate circadian phase-shifting (Brager *et al*, 2010; Ghezzi *et al*, 2013; McElroy *et al*, 2009; Prosser and Glass, 2009; Ruby *et al*, 2009; Seggio *et al*, 2009). In this study, animals consumed far less alcohol (and for a shorter period of time) than the study by Chen *et al* (2004), showing chronic ethanol liquid diet alters SCN *rPer2* gene expression or the study by Seggio *et al* (2009), showing chronic (no choice) ethanol consumption alters circadian activity rhythms; thus, we would not have expected changes in circadian gene expression in the SCN or altered circadian rhythms with this paradigm. It will be important to pursue how SCN *Clock* expression is altered in future studies, although it is difficult under these conditions to do so. All experiments were completed under light/dark (12 : 12) conditions (where both WT and *Clock* mutants show entrainment under these conditions) and none of our experiments were performed under dark/dark conditions. Although it is possible that *Clock* in other brain regions may modulate ethanol intake, this study focused on the VTA (based on previous findings by Mukherjee *et al*, 2010), where we found that VTA *Clock* expression is sufficient to modulate ethanol intake.

Taken together, our results suggest that decreased CLOCK function results in increased dopaminergic tone via its actions as a transcription factor in the VTA, and this underlies the drug-preferring phenotype, which seems to generalize to multiple classes of drugs of abuse. Moreover, the increased glutamatergic tone in these mice may be important in regulating the sedative/hypnotic properties of alcohol but not the rewarding properties. In addition to *Th* and *Cck*, we are currently investigating other interesting CLOCK target genes that may influence neuronal excitability in reward- and stress-related brain regions.

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