

# Adenosinergic Regulation of Striatal Clock Gene Expression and Ethanol Intake During Constant Light

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Circadian rhythm and sleep disruptions occur frequently in individuals with alcohol use disorders (AUD) and present significant barriers to treatment. Recently, a variant of adenosine transporter, equilibrative nucleoside transporter 1 (ENT1), was associated with the co-occurrence of sleep problems and AUD. We have previously shown that mice lacking ENT1 (ENT1 KO) have reduced adenosine levels in the striatum and drink more alcohol compared with wild types (WT). However, it is unknown whether ENT1 deletion disrupts circadian rhythms, which may contribute to alcohol preference in ENT1 KO mice. Here we used these mice to determine whether endogenous adenosine regulates circadian genetic and behavioral rhythms and influences alcohol intake during chronodisruption. We examined circadian locomotor activity in ENT1 KO vs WT littermates and found that ENT1 KO mice were both active earlier and hyperactive compared with WT mice at night. We used real-time PCR and immunohistochemistry to estimate striatal clock gene levels and found that PER2 expression in the striatum was blunted by ENT1 deletion or A2A receptor (A2AR) antagonism. Next, we exposed ENT1 KO and WT mice to constant light (LL) and found further elevation in ethanol intake in ENT1 KO, but not in WT mice, supporting the notion that circadian dysfunction may contribute to increased alcohol intake in ENT1 KO mice. Finally, we showed that A2AR agonist administration normalized PER1 and PER2 expression and circadian locomotor activity in ENT1 KO mice. Together, our results demonstrate that adenosine signaling regulates cellular and behavioral circadian timing and influences alcohol intake during chronodisruption.

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## INTRODUCTION

Circadian rhythm and sleep problems are common, treatment-resistant components of alcohol use disorders (AUD) that greatly contribute to relapse (Brower, 2003; Brower and Perron, 2010). Alcohol is highly disruptive to circadian hormonal and activity rhythms, whereas conversely, circadian desynchrony arising from sleep disorders (Roehrs and Roth, 2001), shiftwork, or repeated jetlag (Gordon *et al*, 1986; Smart, 1979) increases risk of developing AUD. Thus, misalignment of circadian time may be central to the pathophysiology of AUD (Rosenwasser, 2010).

At the cellular level, circadian timing is determined by oscillatory loops of transcription and translation of clock genes (Lowrey and Takahashi, 2004). A central role for the circadian system in AUD is underscored by evidence linking most clock genes with AUD (Albrecht *et al*, 2001; Franken

*et al*, 2000; Gamsby *et al*, 2013; Kovanen *et al*, 2010; Le-Niculescu *et al*, 2008; Lopez-Molina *et al*, 1997; Melendez *et al*, 2011; Perreau-Lenz *et al*, 2012; Spanagel *et al*, 2005; Steinlechner *et al*, 2002; Wang *et al*, 2012; Yang *et al*, 2012). In addition to ethanol (Melendez *et al*, 2011), morphine (Hood *et al*, 2011) and amphetamine (Wongchitrat *et al*, 2013) alter circadian variation in clock gene expression in the mesolimbic reward system during chronic use and/or withdrawal, which may contribute to addictive behavior and/or circadian misalignment. However, little is known about clock gene regulation outside of the suprachiasmatic nucleus (SCN) circadian clock.

Adenosine signaling has an essential role in acute ethanol intoxication, including ataxia, anxiolysis, and sedation, while long-term ethanol exposure leads to neuroadaptation in adenosine signaling that contributes largely to alcohol dependence (Asatryan *et al*, 2011; Dunwiddie and Masino, 2001; Ruby *et al*, 2010). Adenosine transporter equilibrative nucleoside transporter 1 (ENT1) is blocked by acute ethanol and downregulated by chronic ethanol exposure (Nagy *et al*, 1990; Nagy *et al*, 1989), and studies associate ENT1 with AUD in humans (Gass *et al*, 2010; Kim *et al*, 2011). Mice lacking ENT1 (ENT1 KO) display high tolerance to ethanol intoxication and drink alcohol excessively (Choi *et al*, 2004)

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because of low adenosine tone in the striatum (Nam *et al*, 2011; Wu *et al*, 2010). Recent evidence links a variant of ENT1 with circadian sleep disruption and AUD (Gass *et al*, 2010).

As a regulator of circadian timing (Antle *et al*, 2001; Hallworth *et al*, 2002; Sigworth and Rea, 2003), sleep (Halassa *et al*, 2009), and alcohol intake (Asatryan *et al*, 2011; Dunwiddie and Masino, 2001; Ruby *et al*, 2010), adenosine signaling may contribute to chronodisruption in AUD. Adenosine modulates both photic (light) and nonphotic (behavioral) circadian clock resetting (Antle *et al*, 2001; Hallworth *et al*, 2002; Sigworth and Rea, 2003). Caffeine, an adenosine A1 receptor (A1R)/A2A receptor (A2AR) antagonist, lengthens (Oike *et al*, 2011), whereas ethanol shortens (Seggio *et al*, 2009), free-running (endogenous) circadian period. Circadian variation in astrocytic ATP release, extracellular adenosine concentration, and ENT1 and A1R expression has been reported in sleep/wake-promoting areas of the brain (Alanko *et al*, 2003; Marpegan *et al*, 2011; Murillo-Rodriguez *et al*, 2004; Virus *et al*, 1984). Together, this evidence suggests that adenosine may regulate cellular circadian timekeeping.

To explore a role for endogenous adenosine in regulating circadian genetic and behavioral rhythms, we examined circadian entrainment (locomotor activity under a 12:12 light:dark, or LD, photocycle) and striatal clock gene expression in adenosine-challenged ENT1 KO mice (Nam *et al*, 2011) and wild-type (WT) littermates. We then measured ethanol intake in ENT1 KO and WT mice during constant light (LL), a circadian challenge. Finally, we investigated whether selective inhibition of A1R or A2AR in WT mice mimicked some of the clock gene expression changes we observed in ENT1 KO mice, and whether activation of A2AR improved circadian rhythms in ENT1 KO mice.

## MATERIALS AND METHODS

### Animals

ENT1 KO mice were generated (Choi *et al*, 2004). We used F2 hybrid ENT1 KO and WT littermates when they reached 10 weeks old, with a C57BL/6J  $\times$  129  $\times$  1/SvJ background (Crusio *et al*, 2009) to avoid strain-specific influences on behavior or gene expression. Mice were housed in standard Plexiglas cages with food and water available *ad libitum*. The chamber was maintained on 12 h light (500 lux) and 12 h dark (<0.5 lux) cycle (LD) except during constant light (LL; see below). Animal care and handling procedures were approved by Mayo Clinic IACUC according to NIH guidelines.

### Circadian Activity and Drinking

Mice under LD were individually caged and their circadian locomotor rhythms were monitored using infrared sensors interfaced with Clocklab (Coulbourn Instruments, Whitehall, PA). For 2 weeks, mice of each genotype were acclimated to lickometers (Coulbourn Instruments) to visualize circadian drinking patterns. After the acclimation period, mice received ethanol in two-bottle choice (see below); mice using lickometers received ethanol solutions in

the lickometer for the duration of the experiment. After 2 weeks at 10% ethanol in LD, mice were exposed to LL for 2 weeks.

Entrainment to the LD photocycle was determined by calculating the daily activity onset relative to Zeitgeber Time 12 (ZT12), the beginning of the dark phase (active phase for nocturnal rodents). Onset was defined as the first 10 min activity that (1) exceeded 10% maximum daily rate; (2) was preceded by  $\geq 4$  h inactivity; and (3) was followed by  $\geq 30$  min sustained activity. Offset was defined as the final 10 min that was preceded by  $\geq 60$  min sustained activity and followed by  $\geq 4$  h inactivity. Alpha (the active phase) was calculated as the period between activity onset and activity offset (h). Activity distribution was assessed using activity bout analysis (Ruby *et al*, 2009). A bout was defined as a period of continuous activity (regardless of duration) separated by at least 10 min of inactivity. Activity duration (min of activity) and intensity (activity counts) were summed over the active phase (night) and over the inactive phase (day), and averaged over 8 days at each condition (basal, ethanol, and constant light) for each animal.

### Quantitative, Real-Time PCR

Mice were anesthetized with CO<sub>2</sub> and rapidly decapitated at ZT6, ZT10, ZT14, or ZT18. nucleus accumbens (NAc) was isolated under a surgical microscope. RNA was isolated using the RNeasy-Plus Mini kit (Qiagen, Valencia, CA). Real-time, quantitative RT-PCR was performed with the iCycler IQ real-time PCR detection system (Bio-Rad, Hercules, CA) using QuantiTect SYBR Green RT-PCR Kit (Qiagen; (Wu *et al*, 2010). Gene-specific primers were purchased (Qiagen). Protocol: reverse transcription (30 min, 50 °C), denaturation (15 min, 95 °C), and 45 amplification and quantification cycles (15 s, 94 °C; 10 s, 55 °C; and 30 s, 72 °C), each with 1 fluorescence measurement. mRNA expression was normalized by GAPDH. Percentage changes were calculated by subtracting GAPDH C<sub>t</sub> values from C<sub>t</sub> values for the gene of interest (Livak and Schmittgen, 2001).

### Immunohistochemistry

Mice were anesthetized with pentobarbital (80 mg/kg) at ZT22 (peak for PER2 protein in the striatum) and transcardially perfused with 4% paraformaldehyde (Sigma-Aldrich) in PBS. Brains were removed and post-fixed for 24 h in the same fixative at 4 °C. Brains were immersed in 30% sucrose for 24 h, frozen, and cut in 40  $\mu$ m sections using a cryostat (Leica). Free-floating sections were incubated in 50% ethanol, then 10% normal donkey serum in PBS for 30 min, then antibodies against PER2 (1:250, Santa Cruz), and NeuN (1:100, Millipore) or GFAP (1:300, Cell Signaling) overnight. Sections were then incubated in 2% normal donkey serum in PBS for 10 min followed by Alexa 488-conjugated secondary goat anti-rabbit (for PER2, 1:1000, Cell Signaling) and Alexa 555-conjugated goat anti-mouse (for GFAP or NeuN, 1:1000, Cell Signaling) for 2 h. Images from each brain region of interest (NAc core, NAc shell, DMS, DLS, and SCN) were obtained using a LSM 510 confocal laser scanning microscope (Carl Zeiss). Areas of PER2 expression within regions of interest (450  $\times$  450  $\mu$ m) were quantified using NIH Image J software. Quantitative

analysis was performed using a selected region-of-interest background correction method and calculated as a percentage of WT.

### Pharmacological Treatment

For gene expression experiments, C57BL/6J mice were treated with either DPCPX (6.0 mg/kg, *i.p.*; Tocris), ZM-241385 (20.0 mg/kg, *i.p.*; Tocris), or vehicle (15% DMSO, 15% Cremaphor in 0.9% NaCl) for 3 days at ZT22 (peak for PER proteins in the NAc). ENT1 KO and WT mice were treated with CGS-21680 (2.0 mg/kg; Tocris) or vehicle for 3 days at ZT22. On day 4 at ZT14 (peak for Per mRNA in the NAc), mice were anesthetized with CO<sub>2</sub>, rapidly decapitated, and the NAc was isolated and frozen until real-time PCR was performed. For behavioral experiments, a separate group of ENT1 KO and WT mice was treated with CGS-21680 (1.0 mg/kg) or vehicle for 5 days at ZT22, and their behavior was analyzed using Clocklab. Drug doses and treatment length (3–5 days) were selected based on antidipsotropic efficacy (Nam *et al.*, 2013); timing of doses and killing is shown in Figure 4a.

### Ethanol Self-Administration

Oral alcohol self-administration and preference were examined using two-bottle choice (Lee *et al.*, 2013). Mice had 24 h access to two bottles, one containing tap water and the other containing ethanol. The ethanol concentration was raised every fourth day from 3 to 6 to 10% (*v/v*) ethanol. After 2 weeks at 10% ethanol, mice were exposed to constant light (LL) for 2 weeks. A separate group was maintained on 10% ethanol for 4 weeks in LD to control for length of ethanol exposure. Fluid intake and body weight were measured every 48 h to calculate average ethanol consumption (g/kg/day) and preference (% ethanol solution/total fluid consumption).

### Statistical Analyses

Student's *t*-test or one-way ANOVA was used to compare circadian activity and gene expression within a single time point between genotypes or treatments. Two-way ANOVA was used to examine circadian gene expression between genotypes or behavioral response to drugs. Two-way RM ANOVA was used to compare ethanol intake between genotypes during LD *vs* LL. ANOVA were followed by Tukey *post hoc* tests where interactions were found. Results were significant where  $P < 0.05$ .

## RESULTS

### Early Onset, Long Active-Phase and Nighttime Hyperactivity in ENT1 KO Mice

To explore the effect of endogenous adenosine concentration on circadian entrainment and activity level, we analyzed daily activity onset relative to ZT12 (beginning of the dark phase), alpha (active-phase length), and the duration and intensity of activity bouts during the night *vs* day in adenosine-challenged ENT1 KO mice *vs* WT littermates. Representative actograms from WT and ENT1

KO mice are shown in Figure 1a. In contrast to WT, ENT1 KO mice had a positive phase angle of entrainment in LD, becoming active  $\approx 20$  min before ZT12 ( $t = 3.376$ ,  $P = 0.003$ ; Figure 1b). Alpha was longer in ENT1 KO mice ( $t = 3.381$ ,  $P = 0.003$ ; Figure 1c). ENT1 KO mice showed greater 24 h activity duration ( $t = 4.059$ ,  $P < 0.001$ ) and intensity ( $t = 2.419$ ;  $P = 0.025$ ) owing to increased nighttime duration ( $t = 4.643$ ,  $P < 0.001$ ; Figure 1d) and intensity ( $t = 2.519$ ;  $P = 0.020$ ; Figure 1e), as daytime activity duration and intensity were similar between genotypes. These data indicate that ENT1 KO mice are hyperactive at night.

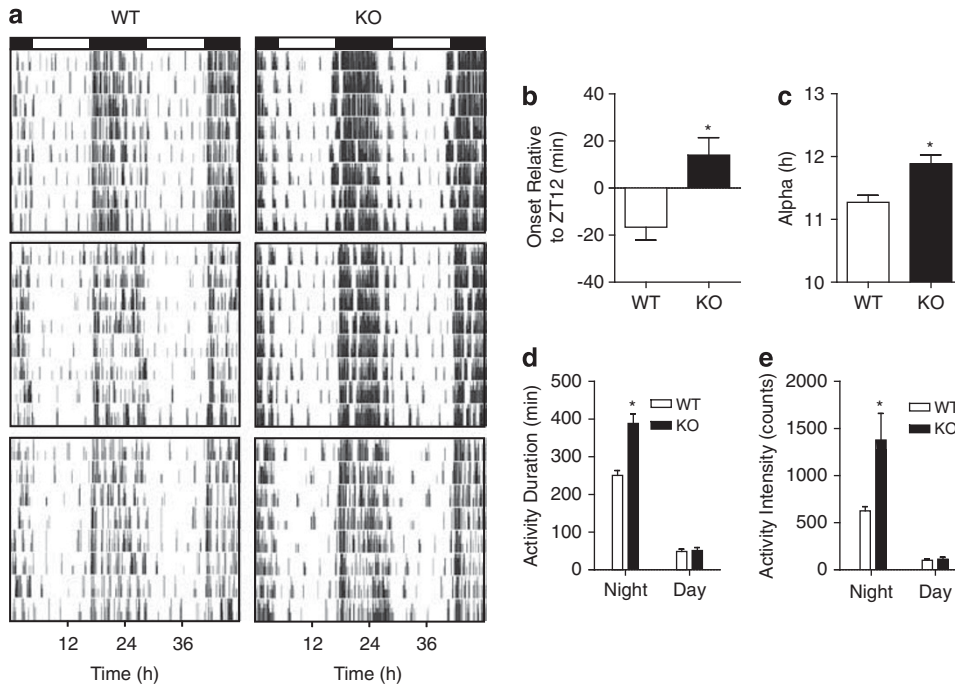
### Altered Clock Gene Expression in the ENT1 KO Striatum

Clock gene expression in the striatum participates in the timing and intensity of daily locomotor activity (Iijima *et al.*, 2002), yet little is known about striatal clock gene regulation. We found that circadian expression of Clock and Dbp was altered in ENT1 KO mice (Supplementary Figure S1), but the most striking difference was in Per2. Two-way ANOVA indicated a main effect of genotype ( $F_{1,38} = 13.617$ ,  $P < 0.001$ ). A time-of-day effect indicated that Per2 expression was circadian ( $F_{3,38} = 14.230$ ,  $P < 0.001$ ). A genotype  $\times$  time-of-day interaction ( $F_{3,38} = 4.117$ ,  $P = 0.014$ ) and subsequent *post hoc* testing revealed 60% reduction in peak Per2 mRNA levels in the ENT1 KO *vs* WT NAc at ZT14 ( $P < 0.001$ ; Figure 2a).

Immunofluorescence confirmed that peak PER2 protein levels (which occur at ZT22,  $\approx 8$  h after peak Per2 gene levels) were also reduced, both in the NAc ( $t = 3.153$ ,  $P = 0.004$ ) and caudate-putamen (CPU;  $t = 3.741$ ,  $P < 0.001$ ; Figure 2b). Background-corrected PER2 immunofluorescence-integrated density averaged  $9.0 \times 10^6 \pm 3.8 \times 10^5$  in WT and  $6.9 \times 10^6 \pm 4.6 \times 10^5$  in ENT1 KO mice in the NAc, and  $8.4 \times 10^6 \pm 3.4 \times 10^5$  *vs*  $6.6 \times 10^6 \pm 3.2 \times 10^5$  in the CPU, respectively. Confocal images showing reduced PER2 immunofluorescence in the striatum are shown in Figure 2c. Colocalization of PER2 with NeuN but not with GFAP (Supplementary Figure S2a) indicated that PER2 was neuronal. No genotype difference in PER2 immunofluorescence was seen in the SCN (Supplementary Figure S2b).

### Chronodisruption-Induced Escalation in Ethanol Intake by ENT1 KO Mice

If circadian misalignment influences the baseline heavy-drinking phenotype of ENT1 KO mice (Choi *et al.*, 2004), then chronodisruption should further increase their ethanol consumption. Higher baseline ethanol intake in ENT1 KO mice during training was confirmed (Supplementary Figure S3). Following 2 weeks at 10% ethanol, mice were exposed to 2 weeks LL. ENT1 KO mice consumed more ethanol ( $F_{1,103} = 4.731$ ,  $P < 0.034$ ), while a genotype  $\times$  photoperiod interaction ( $F_{1,103} = 10.736$ ,  $P = 0.002$ ) and *post hoc* testing revealed increased drinking ( $P = 0.002$ ) by ENT1 KO mice and a genotype difference ( $P = 0.006$ ) during LL (Figure 3a). A genotype  $\times$  photoperiod interaction on ethanol preference ( $F_{1,103} = 8.355$ ,  $P = 0.006$ ) and *post hoc* testing showed that ethanol preference increased during LL only in ENT1 KO mice ( $P = 0.003$ ; Figure 3b). Representative, double-plotted drinkograms (circadian ethanol drinking patterns) are shown in Figure 3c. No changes in ethanol



**Figure 1** Adenosine transporter equilibrative nucleoside transporter 1 (ENT1) regulates circadian locomotor activity. (a) Representative actograms from wild-type (WT; left side) and ENT1 KO mice (right side). (b) ENT1 KO mice had a positive phase angle of entrainment relative to WT, becoming active before lights were turned off (Zeitgeber Time 12, ZT12). (c) ENT1 KO mice had longer alpha (active phase) relative to WT. (d) Increased nighttime, but not daytime, activity duration in ENT1 KO mice compared with WT. (e) Higher nighttime, but not daytime, activity intensity in ENT1 KO mice compared with WT. \* $P < 0.05$  by unpaired, two-tailed *t*-test;  $n = 12$  per genotype. Data are expressed as mean  $\pm$  SEM.

intake occurred in ENT1 KO or WT mice held in LD for the same duration (Lee *et al*, 2013). These data indicate that LL-induced chronodisruption further increases ethanol drinking in ENT1 KO mice.

### A2AR Regulates Per Expression and Circadian Behavior in ENT1 KO Mice

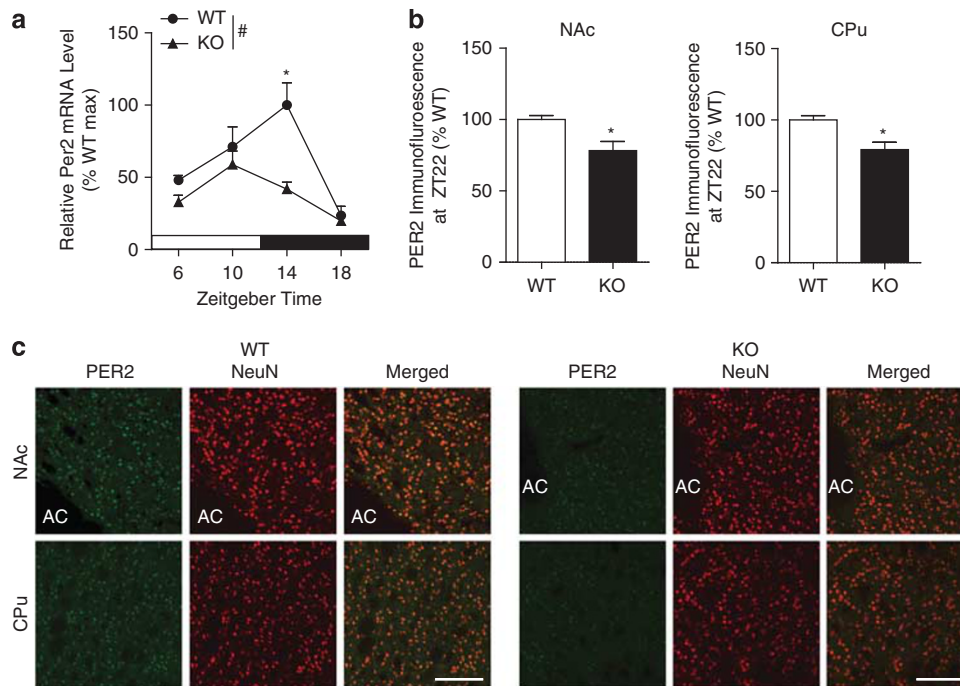
If an adenosine deficit decreases striatal Per expression in ENT1 KO mice, then inhibiting adenosine receptors in WT mice may mimic this change. Pharmacological agents were given for 3 days at ZT22 (PER protein peak); killing took place at ZT14 (per mRNA peak; Figure 4a). Treatment with A1R antagonist DPCPX reduced Per1 expression in the NAc ( $F_{1,23} = 7.906$ ,  $P = 0.003$ ) vs vehicle. A1R antagonist DPCPX ( $P = 0.005$ ) and A2AR antagonist ZM-241385 ( $P < 0.001$ ) decreased Per2 expression ( $F_{1,21} = 12.816$ ,  $P < 0.001$ ) vs vehicle ( $F_{1,21} = 12.816$ ,  $P < 0.001$ ; Figure 4b).

A2AR is enriched in the striatum and its inhibition by caffeine in the NAc produces locomotor activation (Lazarus *et al*, 2011). Increased alcohol consumption (Spanagel *et al*, 2005) and advanced entrainment (Brager *et al*, 2011) in Per2 mutant mice suggest that low Per2 expression may account for early entrainment in ENT1 KO mice. As inhibition of A2AR selectively downregulated Per2 expression, we used A2AR agonist CGS-21680 to determine whether A2AR activation would increase Per2. Three-day CGS-21680 treatment increased Per2 levels similarly ( $\approx 45\%$ ) in ENT1 KO and WT mice, but increased Per1 only in ENT1 KO mice relative to vehicle-treated WT controls ( $t = -3.229$ ,  $P = 0.012$ ; Figure 4c).

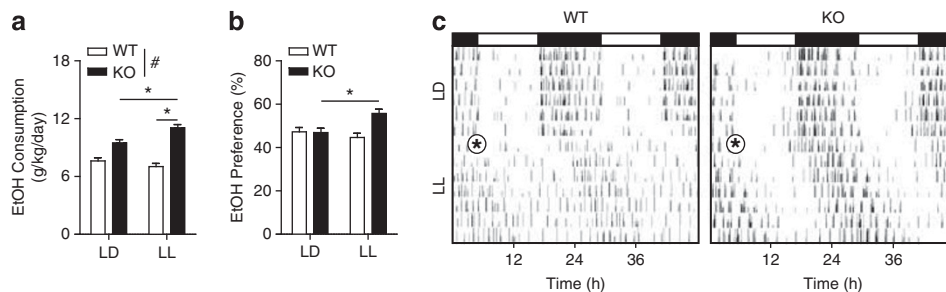
If compromised A2AR-mediated striatal Per expression underlies circadian changes in ENT1 KO mice, then increasing Per expression with CGS-21680 should normalize behavior. As expected, we found main effects of genotype ( $F_{1,21} = 8.671$ ,  $P = 0.009$ ), 5-day CGS-21680 treatment ( $F_{1,21} = 13.210$ ,  $P = 0.002$ ), and a genotype  $\times$  treatment interaction ( $F_{1,21} = 8.783$ ,  $P = 0.008$ ). *Post hoc* analysis revealed that CGS-21680 delayed onset in ENT1 KO ( $P < 0.001$ ) but not in WT mice. Both genotypes responded similarly to vehicle, but differentially to CGS-21680 ( $P = 0.001$ ; Figure 4e). Main effects of genotype ( $F_{1,20} = 6.634$ ,  $P = 0.020$ ), treatment ( $F_{1,20} = 42.613$ ,  $P < 0.001$ ), and a genotype  $\times$  treatment interaction ( $F_{1,20} = 5.454$ ,  $P = 0.032$ ) were also shown for alpha. *Post hoc* analysis showed that CGS-21680 reduced alpha in ENT1 KO ( $P < 0.001$ ) and WT mice ( $P = 0.005$ ), albeit to a greater degree in the former ( $P = 0.004$ ; Figure 3f). Representative actograms of mice from each genotype  $\times$  treatment are shown in Figure 4d. These data support a role for striatal A2AR-mediated Per expression in circadian activity.

## DISCUSSION

We provide strong evidence that adenosine signaling regulates circadian clock gene expression in the striatum. We also demonstrate that endogenous adenosine levels regulate circadian entrainment, nightly activity level, and alcohol intake during chronodisruption. Our study supports a role for adenosine in maintaining circadian timing in reward



**Figure 2** Equilibrative nucleoside transporter 1 (ENT1) regulates Per2 expression in the striatum. (a) ENT1 KO mice showed a reduction in Per2 mRNA in the nucleus accumbens (NAc) during the normal, wild-type (WT) peak time (Zeitgeber Time 14, ZT14) compared with WT littermates  $^{\#}P < 0.05$  for main effect of genotype by two-way ANOVA;  $*P < 0.05$  by *post hoc* Tukey test;  $n = 5$  per genotype. (b) The normal WT peak in PER2 protein expression (ZT22) was also reduced in the NAc and caudate–putamen (CPu) of ENT1 KO mice;  $*P < 0.05$  by unpaired, two-tailed *t*-test;  $n = 8$  per genotype. (c) Representative confocal images showing reduced PER2 immunofluorescence at ZT22 in the NAc and CPu of WT (left) vs ENT1 KO mice (right). Colocalization of PER2 with NeuN indicated that PER2 was expressed in neurons. Scale bar, 100  $\mu\text{m}$ . Data are expressed as mean  $\pm$  SEM.



**Figure 3** Escalation in ethanol intake by equilibrative nucleoside transporter 1 (ENT1) KO mice during a constant light (LL) photocycle. (a) ENT1 KO mice showed increased ethanol consumption during LL compared with LD, whereas wild-type (WT) mice consumed the same amount of ethanol in LL and LD. (b) ENT1 KO mice also showed increased ethanol preference in LL compared with LD, whereas no such change occurred in WT. (c) Representative, double-plotted drinkograms showing circadian drinking patterns from WT and ENT1 KO mice during LD and LL (stars denote beginning of LL).  $^{\#}P < 0.05$  for main effect of genotype by two-way, repeated measures ANOVA;  $*P < 0.05$  by *post hoc* Tukey test;  $n = 27$  per genotype. Data are expressed as mean  $\pm$  SEM.

circuitry and for striatal clock genes in regulating alcohol intake and activity.

The hyperactive phenotype of ENT1 KO mice is consistent with their low adenosine tone and hyperlocomotion produced by caffeine, the latter mediated by inhibition of A2AR in the NAc (El Yacoubi *et al*, 2000; Lazarus *et al*, 2011). In line with increased ethanol intake by A2AR KO mice (Naassila *et al*, 2002), ENT1 deletion also induces striatal A2AR signaling deficits that increase operant reward responding (Nam *et al*, 2013). In contrast, A2AR KO mice show lower exploratory activity, which may reflect the lack of congruence between constitutive and temporary

reduction in A2AR signaling or differences between locomotor measures. In this regard, the present findings illustrate the pitfalls of relying on open-field locomotor testing, which is typically brief ( $\leq 1$  h) and performed during the daytime, when rodents sleep. The present data provide a relevant example: genotype differences in activity only occurred at night, whereas daytime circadian and open-field activity is comparable in ENT1 KO and WT mice (Chen *et al*, 2007; Choi *et al*, 2004; Ruby *et al*, 2011). As cellular timing controls the expression of many genes and proteins (Lowrey and Takahashi, 2004; Miller *et al*, 2007), the time of day at which experiments are performed may be critical in

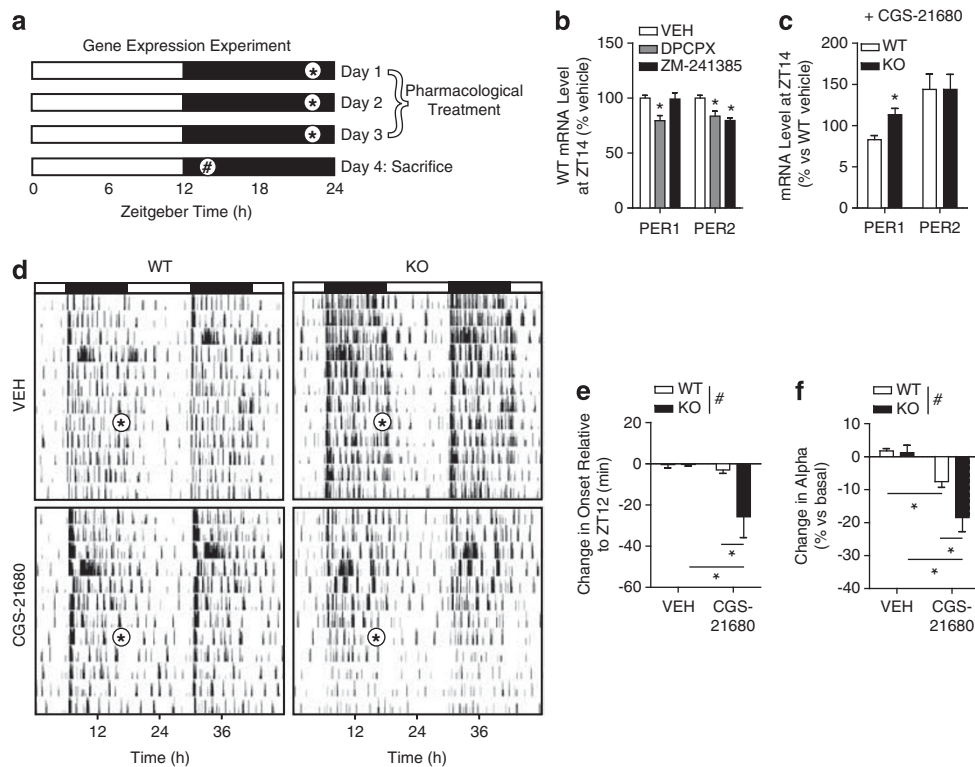
determining the contribution of genes and proteins to behavior.

Per2 mRNA and protein deficits in ENT1 KO mice are intriguing in light of the resemblance ENT1 KO mice bear to Per2 mutants. Both are well-established animal models of glutamatergic dysregulation and compromised astrocyte function in AUD (Choi *et al*, 2004; Lee *et al*, 2013; Nam *et al*, 2011; Spanagel *et al*, 2005). Early awakening in ENT1 KO mice is also consistent with the role of Per2 in circadian phase regulation (Albrecht *et al*, 2001). The circadian phenotype in Per2 mutants is severe; they become active 2 h before lights off (Bode *et al*, 2011; Brager *et al*, 2011), whereas ENT1 KO were active  $\approx$  20 min before ZT12. Per2 mRNA expression in ENT1 KO mice was also advanced relative to WT (ZT10 vs ZT14, respectively), which could indicate that peak PER2 protein expression occurred early. However, Per2 mRNA levels in ENT1 KO mice at ZT10 were still substantially lower than the ZT14 peak for WT mice. Given that PER proteins inhibit their transcripts, examining 24 h striatal PER2 protein expression in ENT1 KO mice may be informative.

ENT1 expression is very low in the hypothalamus (Guillen-Gomez *et al*, 2004), and therefore may not be a

significant source of extracellular adenosine in the SCN. Thus, ENT1 deletion would not be expected to greatly alter SCN adenosine tonus. Although outside the scope of this study, proper assessment would require examining SCN adenosine levels, 24 h SCN PER2 expression, and phase-resetting responses to A1R-gated photic input (Sigworth and Rea, 2003) in ENT1 KO mice. However, the present data support a role for striatal adenosine and Per2 in the observed genotype difference in behavior, as SCN PER2 expression was comparable between genotypes. Moreover, activation of A2AR, which is enriched in the striatum but scant in the hypothalamus, reversed the deficits of ENT1 KO mice. In this regard, other evidence exists for clock genes in reward circuitry contributing to circadian amplitude (Mukherjee *et al*, 2010).

Our study indicates that cellular circadian timing in the striatum may influence alcohol consumption and preference, particularly during chronodisruption. LL usually lengthens circadian period (Figure 3c) and dampens Per expression (Ohta *et al*, 2005). Long-term LL (50+ days) constitutively elevates PER2 protein in the murine SCN, which negatively regulates Per2 transcripts (Munoz *et al*, 2005). Thus, the fact that LL did not change ethanol



**Figure 4** A2A receptor (A2AR)-mediated regulation of Per gene expression and circadian behavioral rhythms in equilibrative nucleoside transporter 1 (ENT1) KO mice. (a) Diagram depicting times of treatment with adenosine receptor ligands (Zeitgeber Time (ZT22); wild-type (WT) peak for PER protein expression in striatum) and killing (ZT14; WT peak for Per gene expression in striatum). (b) Three-day treatment of A1R antagonist DPCPX (6.0 mg/kg, i.p.) or A2AR antagonist ZM-241385 (20.0 mg/kg, i.p.) at ZT22 (late night) differentially affected clock gene expression in WT mice, with the former downregulating peak expression of Per1 and Per2, while the latter only reduced expression of Per2; \* $P < 0.05$  by one-way ANOVA;  $n = 6$  per treatment. (c) Three-day treatment of A2AR agonist CGS-21680 (2.0 mg/kg) upregulated Per1 only in ENT1 KO mice and Per2 in both WT and ENT1 KO mice compared with vehicle-treated WT controls;  $P < 0.05$  by unpaired, two-tailed *t*-test;  $n = 6$  per treatment. (d) Representative actograms from WT (left side) and ENT1 KO (right side) mice treated with vehicle (top) or A2AR agonist CGS-21680 (bottom) for 5 days. Stars denote daily treatment time at ZT22; for simplicity, only the first treatment day is starred. (e) Five-day treatment with CGS-21680 (1.0 mg/kg, i.p.) at ZT22 (late night) delayed activity onset in ENT1 KO, but not in WT mice. (f) Alpha was reduced by 5-day CGS-21680 treatment in both ENT1 KO and WT mice, but to a greater degree in the former; # $P < 0.05$  for main effect of genotype by two-way ANOVA; \* $P < 0.05$  by *post hoc* Tukey test;  $n = 5$  per genotype  $\times$  treatment. Data are expressed as mean  $\pm$  SEM.

consumption or preference in WT mice indicates that disruption of SCN timing alone is insufficient to affect drinking. However, the additive effect of LL-induced chronodisruption on ethanol intake in ENT1 KO mice validates the idea that pre-existing circadian misalignment may increase ethanol consumption during environmental challenges to the circadian system.

Our findings implicate ENT1 and adenosine receptor-mediated signaling in alcohol-induced flattening of Per gene rhythms (Chen *et al*, 2004; Huang *et al*, 2010). Specifically, A1R inhibition downregulated Per1 and Per2 expression, whereas A2AR inhibition downregulated only Per2. However, A2AR agonist CGS-21680 treatment in Per1/Per2-deficient ENT1 KO mice increased expression of both genes, indicating that A2AR affects Per1 levels as well. Adenosinergic regulation of NAc clock genes has important implications regarding the influence of A1R/A2AR antagonist caffeine on alcohol consumption. Reduced sensitivity to intoxication may underlie the frequently toxic blood alcohol concentration achieved by consuming caffeinated alcoholic beverages (Attwood, 2012; Attwood *et al*, 2012). Here, A2AR antagonism only once a day for 3 days was sufficient to reduce striatal Per2, which itself increases alcohol intake (Spanagel *et al*, 2005). Furthermore, the dose of A2AR antagonist ZM-241385 we used herein increases alcohol intake (Nam *et al*, 2013).

As A2AR is enriched in the striatum, the observed A2AR-mediated improvements in Per expression and circadian behavior in ENT1 KO mice argue that the striatal cellular clock participates in the timing and intensity of daily activity. The dose range of CGS-21680 used also reduces alcohol drinking in mice (Nam *et al*, 2013), implying a relationship between circadian rhythm improvement and decreased drinking. Similar to several studies (Houchi *et al*, 2013; Jones *et al*, 2012; Knapp *et al*, 2001; Rimondini *et al* 1997), we observed that CGS-21680 reduced locomotor activity. Alpha (active-phase duration) was reduced in CGS-21680-treated mice of both genotypes *vs* vehicle-treated WT controls (Figure 4d). This is likely related to late-night treatment timing, which coincided with peak PER2 protein expression, but may have reduced late-night locomotion (Figure 4d and e). However, alpha decreased more markedly in ENT1 KO *vs* WT mice, as CGS-21680 delayed onset only in ENT1 KO mice. As CGS-21680 was administered during the day in Nam *et al* (2013), it is also unlikely that hypolocomotion underlies reduced ethanol drinking in that study.

Our results suggest a role for striatal adenosine signaling in psychiatric disorders with a prominent hyperactivity component and animal models thereof. Of note, adult ADHD is linked with blunted Per2 expression (Baird *et al*, 2012). It is also interesting that the other clock genes with altered circadian profiles in ENT1 KO mice, Clock and Dbp, are associated with ADHD (Kissling *et al*, 2008; Roybal *et al*, 2007; Xu *et al*, 2010), bipolar disorder (Le-Niculescu *et al*, 2008; Lee *et al*, 2010; Shi *et al*, 2008; Soria *et al*, 2010), anxiety (Le-Niculescu *et al*, 2011a), and AUD (Le-Niculescu *et al*, 2011b; Le-Niculescu *et al*, 2008). Interestingly, ENT1 KO mice display mania-like behavior (Ruby *et al*, 2011) similar to Dbp KO mice (Le-Niculescu *et al*, 2008). Stress-reactive Dbp KO mice show reduced prefrontal cortical EAAT2 (Le-Niculescu *et al*, 2008) consistent with the ENT1

KO striatum (Lee *et al*, 2013). Thus, future studies examining a role for Dbp in ENT1 KO mice are warranted.

Our findings reveal that striatal A2AR-mediated signaling may influence cellular and behavioral circadian timing and activity level. Although clock timing in the SCN itself does not appear to change alcohol intake, compromised reward network timing may confer susceptibility to increased drinking during chronodisruption. This has important implications for individuals who experience chronic environmental challenges to the circadian system, as in shiftwork, jetlag, and sleep disruption. Our study also implies that striatal dysfunction, which adversely affects mood and drives drug intake, may partly underlie the high prevalence of circadian misalignment in psychiatric and substance use disorders.

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The authors declare no conflict of interest.

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