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Type 1 equilibrative nucleoside transporter (ENT1) regulates sex-specific ethanol drinking during disruption of circadian rhythms

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Abstract

Disruptions in circadian rhythms are risk factors for excessive alcohol drinking. The ethanol-sensitive adenosine equilibrative nucleoside transporter type 1 (ENT1, *slc29a1*) regulates ethanol-related behaviors, sleep, and entrainment of circadian rhythms. However, the mechanism underlying the increased ethanol consumption in ENT1 knockout (KO) mice in constant light (LL) and whether there are sex differences in ethanol consumption in ENT1 mice are less studied. Here, we investigated the effects of loss of ENT1, LL and sex on ethanol drinking using two-bottle choice. In addition, we monitored the locomotor activity rhythms. We found that LL increased ethanol drinking, reduced accumbal ENT1 expression and adenosine levels in male, but not female mice, compared to control mice. Interestingly, only LL-exposed male, not female, ENT1 KO mice exhibited higher ethanol drinking and a longer circadian period with a higher amplitude compared to wildtype (WT) mice. Furthermore, viral-mediated rescue of ENT1 expression in the NAc of ENT1 KO mice reduced ethanol drinking, demonstrating a possible causal link between ENT1

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Authors contribution

DSC was responsible for the study concept and design and drafted the manuscript. YFJ performed all the data analysis, also helped draft the manuscript and interpret the findings. YFJ and CV contributed to the acquisition of animal data. AMH assisted with data analysis and interpretation of findings. CV, AMH, LP, MV, KW, and AM provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Conflict of interest

Dr. DS Choi is a scientific advisory board member to Peptron Inc. and the Peptron had no role in preparation, review, or approval of the manuscript; nor decision to submit the manuscript for publication. All the other authors declare no biomedical financial interests or potential conflicts of interest.

expression and ethanol drinking in males. Together, our findings indicate that deficiency of ENT1 expression contributes to excessive ethanol drinking in a sex-dependent manner.

Keywords

Adenosine; constant light; ethanol drinking; ENT1; sex; nucleus accumbens

Introduction

Increasing evidence suggests that circadian rhythm disruptions including irregular work schedules or light at night contribute to alcohol use disorder (AUD) in humans[1,2] and animal models[3,4]. At the cellular level, the oscillating transcription-translation feedback loops of circadian genes regulate circadian rhythms[5,6]. Genetic studies in humans and animals have implicated circadian genes in alcohol use[7–9]. Furthermore, preclinical studies have shown that circadian genes regulate midbrain dopaminergic activity and subsequently, drug intake and reward[10].

Adenosine, an inhibitory neurotransmitter and neuromodulator, is implicated in several aspects of circadian rhythms and AUD[11–13]. In humans, a genetic variant of the adenosine transporter 1 (ENT1, *SLC29A1*) is associated with AUD and circadian sleep disruption[14]. Chronic ethanol exposure downregulates ENT1 expression in cultured neuronal cells[15]. Our previous work suggests that a downregulation of ENT1 decreases extracellular adenosine levels as mice lacking ENT1 exhibit reduced extracellular adenosine levels in the striatum as measured from tissue lysate[16] as well as from *in vivo* microdialysis[17]. Mice lacking ENT1 also display increased tolerance to ethanol intoxication and a high preference for ethanol[18]. Congruent with our previous findings, several recent animal studies illustrate that ENT1 expression is inversely correlated with ethanol drinking[19–21].

Adenosine regulates both sleep homeostasis and circadian rhythms[22,23]. Adenosine levels progressively increase during wakefulness and peak prior to sleep[24,25]. Mice lacking ENT1 show decreased non-rapid eye movement sleep during the light period[26], which may be associated with increased ethanol consumption. Studies also suggest that ENT1 regulates circadian rhythms entrainment[27–29]. Moreover, we previously found that ENT1 knockout (KO) mice exhibit down-regulated striatal *Per1* and *Per2* expression, as well as differences in home cage activity rhythms while housed under a 12 h light /12 h dark schedule (LD)[4]. Interestingly, constant light (LL) further escalated ethanol consumption in ENT1 KO mice suggesting that impaired adenosine signaling may increase vulnerability to circadian disruption-mediated increases in ethanol consumption[4]. However, the mechanisms underlying the effects of LL on ethanol consumption in ENT1 KO mice are unclear. We hypothesize that LL disrupts adenosine signaling which may explain the effects of LL on ethanol consumption. In addition, we expect that ENT1 KO mice are more susceptible to LL-induced circadian disruption, which may also contribute to their increased ethanol consumption.

Recently, it has become apparent that sex hormones such as testosterone and estrogen play a major role in circadian activity patterns and alcohol consumption[30–32]. Interestingly, similar

to male ENT1 KO mice, female ENT1 KO mice exhibit increased ethanol drinking compared to WT mice[33]. However, whether there are sex differences in ethanol-related behaviors in response to LL in ENT1 KO mice has not been addressed yet. Thus, in this study, we included both male and female WT and ENT1 KO mice and hypothesize that we will observe sex differences in the effects of LL on circadian rhythms and ethanol consumption.

Materials and methods

Mice

Both male and female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) aged 7–9 weeks old were used in this study. ENT1 KO mice[18]were generated from our lab by crossing F2 hybrid ENT1 KO and WT littermates when they reached 8 weeks old, with a C57BL/6J;129X1/SvJ background[18]. Mice were group-housed (4–5 animals per cage) in standard Plexiglas cages in a temperature (22–24°C) and humidity (50%) regulated environment with access to standard lab food and water *ad libitum*. Mice were housed on a 12-hour/12-hour light (500 lux)/dark (<0.5 lux) cycle (LD) except for during the LL experiment, as described below. Investigators who performed the behavioral experiments and data analysis were blinded from the mouse genotypes and treatments. All animal care, handling procedures and experimental protocols were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) in accordance with the guidelines set forth by the National Institutes of Health.

Two-bottle choice drinking experiment

Ethanol self-administration was examined by a two-bottle choice drinking paradigm[34,35] during which mice had access to a bottle of tap water and a bottle of ethanol diluted with tap water. The ethanol concentration was raised every fourth day from 3% to 6% and finally to 10% (v/v). Fluid intake and body weight were measured every 48 h to calculate average ethanol consumption (g/kg/day) and preference (%).

Quantitative real-time PCR (qRT-PCR)

Mice were euthanized by CO_2 asphyxiation and rapidly decapitated. The nucleus accumbens (NAc), medium prefrontal cortex (mPFC), and hippocampus were immediately isolated under a surgical microscope. Total RNA was isolated using the RNeasy Plus Mini kit (Qiagen; Cat No. 74134) and then reverse transcribed by the Life Technologies Superscript III First-Strand Synthesis SuperMix kit (Cat No. 18080400) to obtain cDNA. The thermal cycling protocol for reverse transcription was 30 min at 50 °C followed by 15 min at 95 °C. Quantitative RT-PCR was performed on CFX 96 Touch™ Real-Time System, C1000 Touch Thermal Cycler (Bio-Rad) using QuantiTect SYBR Green RT-PCR Kit (Qiagen; Cat No. 204143) and gene-specific primers (ENT1, A_1R, $A_{2A}R$, Per1, Per2, GAPDH; Qiagen). The thermal cycling protocol for qRT-PCR was 45 amplification quantification cycles of 15 s at 94 °C, followed by 10 s at 55 °C, and then 30 s at 72 °C. The targeted gene mRNA expression was normalized to GAPDH. Percentage changes were calculated by subtracting GAPDH Ct values from Ct values for the gene of interest using the $2^{-\Delta\Delta Ct}$ method[36].

Circadian activity

The circadian locomotor rhythms were monitored by infrared sensors interfaced with Clocklab (Actimetrics, Wilmette, IL) while the mice were individually caged. The circadian period (tau) under LL was determined by chi-square periodogram analysis in Clocklab. During LL, tau was typically greater than 24 h. After approximately two weeks of LL exposure, the circadian activity became arrhythmic[37,38]. The percentages of mice that were arrhythmic or had a tau greater than 24 h were calculated in each group. We used the amplitude (in AU) as the circadian power that of the circadian rhythm, which is the peak amplitude of the chi-square periodogram from the maximum amplitude value of the power spectrum. AU indicates arbitrary unit[37,38].

Stereotaxic viral injection

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and positioned in a stereotaxic instrument (KOPF Instruments). Either the ENT1 virus (AAV9-D2R-ENT1/eGFP; 1.4×10^{13} GC/ml, 1.0 μl, Vector Biolabs) or control virus (AAV9-D2R-eGFP; 1.4×10^{13} GC/ml, 1.0 μl, Vector Biolabs) was delivered by 35-gauge syringe needles (World Precision Instruments) to the NAc bilaterally (AP: +1.3 mm, ML: ± 1.0 mm, DV: −4.25 mm) at a rate of 0.1 μl/min for 10 min. At the end of injection, needles remained in place for 5 min to ensure complete delivery of the viral bolus and were then slowly retracted to minimize trauma and viral spreading. The scalp was sutured with 5–0 polyviolene sutures (Sharpoint). Behavioral tesing began 3 weeks after surgery to allow for sufficient viral expression.

Immunohistochemistry

Mice were euthanized by CO_2 asphyxiation followed by rapid brain removal. Brains were fixed in 4% paraformaldehyde (Sigma Aldrich) for 24 h and then immersed in 30% (w/v) sucrose in 0.1M PBS at 4 °C for 48 h until the tissue sunk. Coronal free-floating sections (40 μm) were cut by a cryostat and incubated in 0.5% Triton-PBS for 15 min and then in 5% (w/v) bovine serum albumin (BSA)-PBS for 1 h at room temperature. Sections were then incubated with rabbit anti-c-Fos primary antibody (1:100, Cat No.: sc-52, Santa Cruz Biotechnology), rabbit anti-D2R (1:100, Cat No.: sc-9113, Santa Cruz Biotechnology), or mouse anti-NeuN (1:1000, Cat No.: Ab10424, Abcam) at 4 °C overnight. After washing with PBS, slices were incubated with secondary antibody (1:500, Alexa 594-conjugated goat anti-rabbit or Alexa 405-conjugated goat anti-mouse; Abcam) for 3 h at room temperature. Images from the brain region of interest (NAc) were obtained on a LSM 510 confocal laser scanning microscope (Carl Zeiss) with a 20X objective. Quantification of c-Fos positive cells in the NAc per mm^2 was carried out using 3–5 coronal sections per mouse, using a minimum of 3 mice per group. The total number of c-Fos positive cells present in the NAc from all sections of each mouse was averaged.

Microdialysis

Animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic instrument (KOPF Instruments). A guide cannula was implanted into the NAc (AP: 1.3 mm; ML: 0.5 mm; DV: −3.5 mm), and mice were allowed to recover

for 7 days recovery as previously described (Chen et al., 2010; Nam et al., 2011). At the time of collection, a microdialysis probe with a 2.0 mm cellulose membrane (Eicom, MW cut off: 50,000 Da) that connected to a microsyringe pump (Eicom) was inserted and secured to the guide cannula. Ringer's solution (145 mM NaCl, 2.7 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH 7.4) was delivered to the probe from the pump at a 0.5 μl/min flow rate. Samples were collected for a continuous 2 h twice daily during the daytime (9–11 AM) and nighttime (7–9 PM), respectively. Microdialysates were immediately frozen after each collection and stored at −80 °C until analyzed. Adenosine level was measured by liquid chromatograph mass spectrometry using a similar method as previously described[39]. Microdialysate samples were spiked with internal standards, deproteinized with cold methanol and followed by centrifugation at 10,000 g for 5 minutes. The supernatant was immediately derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate according to Waters' MassTrak kit. An 11-point calibration curve (0, 0.025, 0.050, 0.250, 1.0, 3.0, 5.0, 15.0, 30.0, 40.0, 80.0 μM) made up of purchased adenosine standards underwent a similar derivatization procedure after the addition of the same internal standards. The derivatized standards and samples were analyzed on a triple quadrupole mass spectrometer (Thermo TSQ Quantum Ultra) coupled with an Ultra Pressure Liquid Chromatography system (Waters Acquity UPLC). Data acquisition was done using a select ion monitor (SRM). Concentrations of each unknown were calculated against their perspective calibration curves.

ENT1 binding assay

To assess the functional ENT1 level, we use nitrobenzylthioinosine (NBTI) to do ENT1 binding assay. For [^{3}H] NBTI binding, mice were deeply anesthetized in CO_2 and then the brain tissues were rapidly removed on ice. The samples were homogenized in 10% sucrose solution and centrifuged at 1,000 g for 4 min. The supernatant was carefully collected and centrifuged again at 10,000 g for another 10 min, and then the pellet was resuspended in the 50 mM Tris-HCl buffer with protease inhibitor. [^{3}H] NBTI saturation binding assays were performed at 37 °C in 10 mM Tris-HCl with non-specific binding determined in the presence of 10 μM Dilazep. Binding reactions were terminated by filtration with ice-cold Tris-HCl buffer through Whatman GF/B paper using a cell harvester (Inotech). Filters were dried and incubated in scintillant, and then the membrane-bound radioligand was measured using scintillation spectrometry. Specific binding was determined by substituting nonspecific binding from total binding values[18,40].

Statistical analysis

All data are expressed as mean ± SEM (standard error of the mean). Fisher's exact test was used to compare categorical distributions between groups. Two-tailed Student's *t*-test was used to compare the difference between two groups. One-way ANOVA was used to compare the differences for one factor (treatment or genotype), followed by Tukey *post hoc* test for pairwise comparisons. Two-way repeated measures ANOVA was used to detect the effects of treatment and genotypes on ethanol consumption and ethanol preference. ANOVA were followed by Tukey *post hoc* tests where interactions were found. Statistical significance was set at $P < 0.05$. All statistical calculations were performed using Graphpad Prism 7 (La Jolla CA, USA).

Results

Constant light increased ethanol drinking and reduced the ENT1 mRNA expression in the nucleus accumbens of male, but not female mice

To investigate the effects of disruption of circadian rhythms in the modulation of ethanol drinking and neuronal activities, as shown in Fig. 1A, C57BL/6J mice were exposed to constant light (LL) or LD for two weeks, which is a simple and straightforward method to disrupt circadian rhythms[41,42]. After two weeks of LL or LD, mice consumed ethanol in a two-bottle choice drinking paradigm under LL or LD (days 14–26, Fig.1A). Male mice exposed to LL exhibited increased ethanol consumption (Fig. 1B) and ethanol preference (Fig. 1C) compared to the control group in a normal LD cycle. Two-way ANOVA indicated a main effect of ethanol concentration and lighting condition on ethanol intake (consumption: lighting condition: $F_{(1,19)} = 6.136$, $P = 0.023$, Fig. 1B; preference: lighting condition: $F_{(1,19)} = 5.018$, $P = 0.037$, Fig. 1C). Interestingly, in female mice, we found no effect of constant light on ethanol consumption (two-way ANOVA, group effect: $F_{(1,18)} = 3.088$, $P = 0.096$; Fig. 1D) or preference (two-way ANOVA, group effect: $F_{(1,18)} = 0.273$, $P = 0.607$; Fig. 1E).

Using a separate cohort of mice, we found that two weeks of LL exposure (Fig. 2A) significantly reduced ENT1 mRNA expression in the NAc of male mice (two-tailed unpaired *t*-test, $t_{(14)} = 2.711$, $P = 0.017$; Fig. 2B), but not in the mPFC (two-tailed unpaired *t*-test, $t_{(10)} = 0.008$, $P = 0.994$; Fig. S1A and B) or hippocampus (two-tailed unpaired *t*-test, $t_{(10)} = 1.769$, $P = 0.107$; Fig. S1A and D) compared to that of LD group, suggesting that reduced accumbal ENT1 is involved in LL-mediated increases in ethanol drinking. In the female mice, LL didn't have an effect on ENT1 mRNA expression in the NAc compared to the LD group (two-tailed unpaired *t*-test, $t_{(16)} = 1.202$, $P = 0.246$; Fig. 2C), but reduced ENT1 mRNA expression in the mPFC (two-tailed unpaired *t*-test, $t_{(9)} = 3.848$, $P = 0.004$; Fig. S1A and C) and had no effect in the hippocampus (two-tailed unpaired *t*-test, $t_{(10)} = 0.885$, $P = 0.397$; Fig. S1A and E).

Constant light decreased adenosine levels and adenosine receptors in the nucleus accumbens of male, but not female mice

We investigated the effect of LL on adenosine levels in the NAc during the daytime (9 AM → 11 AM) and nighttime (7 PM → 9 PM) by *in vivo* microdialysis (Fig. 3A). During the daytime, adenosine levels were significantly lower in LL than in LD groups in the NAc of male mice. However, during nighttime, adenosine levels were not changed between the LL and LD groups in male mice (two-tailed unpaired *t*-test; Daytime: $t_{(20)} = 2.577$, $P = 0.018$; Nighttime: $t_{(15)} = 1.222$, $P = 0.241$; Fig. 3B). Interestingly, in female mice, adenosine levels were similar between the LL and LD groups during both the daytime and nighttime (two-tailed unpaired *t*-test; Daytime: $t_{(16)} = 0.518$, $P = 0.611$; Nighttime: $t_{(13)} = 1.146$, $P = 0.273$; Fig. 3C).

In addition, we examined the effects of LL on the expression of adenosine receptor genes in the NAc specifically in the daytime since the adenosine levels were decreased by LL only in the daytime. In male mice, adenosine A_1 receptor (A_1R) and adenosine A_{2A} receptor

($A_{2A}R$) mRNA levels were significantly reduced in two weeks of LL exposure (two-tailed unpaired *t*-test; A_1R: $t_{(16)} = 3.623$, $P = 0.002$; $A_{2A}R$: $t_{(16)} = 6.741$, $P < 0.0001$; Fig. 3D). In female mice, LL showed no effect on A_1R or $A_{2A}R$ genes expression in the NAc (two-tailed unpaired *t*-test; A_1R: $t_{(16)} = 1.266$, $P = 0.224$; $A_{2A}R$: $t_{(16)} = 0.068$, $P = 0.946$; Fig. 3E). These results suggest that LL-induced decreases in adenosine levels in the NAc during daytime, which may be accompanied by reduction of A_1R and $A_{2A}Rs$ expression levels only in male mice.

Constant light increased ethanol drinking and period of activity rhythms in male, but not in female ENT1 KO mice

In LD, ENT1 KO consumed more ethanol compared to WT mice[17,18]. Here, we examined whether LL alters ethanol drinking in ENT1 KO mice in a sex-dependent manner (Fig. 4A). Male ENT1 KO mice showed higher ethanol consumption (Fig. 4B) and preference (Fig. 4C) than WT mice. Two-way ANOVA indicated a main effect of genotype (Consumption: $F_{(1,19)} = 16.24$, $P = 0.001$, Fig. 4B; Preference: $F_{(1,19)} = 8.582$, $P = 0.009$, Fig. 4C), suggesting that ablation of ENT1 further exacerbates ethanol drinking under LL condition. We then examined the effects in female ENT1 KO mice. No significant difference was found between WT and ENT1 KO female mice in their ethanol consumption (two-way ANOVA, group effect: $F_{(1,18)} = 2.045$, $P = 0.169$, Fig. 4D) or preference (two-way ANOVA, group effect: $F_{(1,24)} = 0.657$, $P = 0.428$; Fig. 4E).

Next, as shown in Fig. 5A, we examined the LL-induced free running pattern in male and female mice since LL generates a typical free-running period longer than 24 hours and eventually causes the mice to become arrhythmic[38,43]. As expected, LL disrupted circadian rhythms by decreasing the amplitude and total activity in both genotypes of male and female mice (Supplementary Table S1; Fig. S2A–D). Interestingly, while male WT mice showed an arrhythmic periodogram after approximately two weeks of LL exposure, ENT1 KO mice exhibited a long period throughout the LL (Fig. 5B). Thus, a higher percentage of ENT1 KO mice had a long circadian period relative to WT mice (WT: 30% *vs* KO: 80% respectively, Fisher's exact test, $P < 0.0001$; Fig. 5C). In addition, the ENT1 KO mice showed higher amplitude rhythms than WT in LL (two-tailed Mann-Whitney *t*-test, $P = 0.045$; Fig. 5D), suggesting that male ENT1 KO mice are less disrupted by LL compared to WT mice. In female mice, however, a similar percentage of ENT1 and WT mice number exhibited a long tau or arrhythmic activity (Fisher's exact test, $P = 0.200$; Fig. 5E and F). Also, the amplitude was similar between female ENT1 KO and WT mice in LL (two-tailed Mann-Whitney *t*-test, $P = 0.986$; Fig. 5G).

Restoring accumbal ENT1 expression rescued LL-dependent ethanol drinking in ENT1 KO mice

To examine the causal relationship between ENT1 expression and LL-dependent ethanol drinking, we determined the effects of viral-mediated ENT1 expression in the NAc of ENT1 KO mice (Fig. 6A). Using the [^{3}H] NBTI binding assays we showed that functional ENT1 levels were increased in the NAc compared to the control virus injected into WT mice (two-way ANOVA, group effect: $F_{(1,10)} = 9.529$, $P = 0.011$; Fig. 6B). The co-localization of eGFP, D2R, and NeuN demonstrated the specificity of virus expression which was

exclusively expressed in D2R-positive neurons in the NAc (Fig. 6C). Additionally, the administration of ENT1 virus (vENT1) into WT mice increased ENT1 mRNA levels, and resulted in similar ENT1 expression levels as WT mice when administration into the ENT1 KO mice (one-way ANOVA, $F_{(2,13)} = 9.792$, $P = 0.003$; Tukey *post hoc* for KO+vENT1 *vs* WT, $P = 0.209$, WT+vENT1 *vs* WT, $P = 0.038$, KO+vENT1 *vs* WT+vENT1, $P = 0.002$; Fig. 6D). Importantly, restoring striatal ENT1 expression in ENT1 KO mice significantly suppressed the increased ethanol consumption compared to the ENT1 KO mice in LL. Two-way ANOVA indicated a main effect of group ($F_{(2,36)} = 11.65$, $P = 0.0001$; followed by Tukey's multiple comparisons test, for WT *vs* KO, $P < 0.0001$; for KO *vs* KO+vENT1, $P = 0.031$; for WT *vs* KO+vENT1, $P = 0.091$; Fig. 6E). The post-hoc analysis of the two-way ANOVA showed significance between WT and KO under 6% (WT *vs* KO, $P = 0.008$) and 10% (WT *vs* KO, $P = 0.001$) of ethanol concentration respectively, and a difference between KO and KO+vENT1 under the 10% EtOH concentration (KO *vs* KO+vENT1, $P = 0.028$). These results suggest that restoring ENT1 in the NAc reversed the effects of constant light on increased ethanol drinking in ENT1 KO mice. However, the administration of ENT1 virus into WT mice had no significant effect on the circadian activity and both groups showed arrhythmic homecage activity after approximately two weeks LL exposure (Fig. S3A). The amplitude and total daily activity of the vENT1 group didn't differ from that of the control group (two-tailed Mann-Whitney *t*-test, amplitude: $P = 0.618$; activity: $P = 0.618$; Fig. S3B and C).

We used c-Fos labeling to study the effects of loss of ENT1 on neuronal activity in the NAc according to the schematic illustration (Fig. S4A). ENT1 KO mice that had increased ethanol drinking during LL, showed significantly decreased c-Fos levels in the NAc compared to WT mice. Whereas, administrating ENT1 virus into the NAc of ENT1 KO mice prevented the decreased c-Fos levels in ENT1 KO mice (one-way ANOVA, $F_{(3,8)} = 14.69$, $P = 0.001$; followed by Tukey *post hoc* tests, for WT *vs* KO, $P = 0.048$; for KO *vs* KO +vENT1, $P = 0.001$; Fig. S4B and C). Taken together with the results of the previous ethanol drinking experiments, our findings demonstrate that decreased c-Fos levels in the NAc may be related to higher ethanol intake in ENT1 KO mice during LL, which can be partially reversed by restoring ENT1 expression in the NAc of male mice.

Discussion

Our findings highlight that hypo-adenosinergic signaling in the NAc promotes ethanol intake. We found that constant light exposure reduces adenosine levels along with adenosine receptors and ENT1, which is attributed to excessive ethanol drinking in male mice, but not in female mice. Alcohol disrupts sleep homeostasis while sleep disruption also worsen alcohol intake[44]. Since ethanol intake promotes sleep, it has been suggested that alcohol use disorder (AUD) patients self-medicate with alcohol to fall asleep, which exacerbates alcohol drinking and existing sleep disturbances[45]. Interestingly, microinjection of a selective adenosine A_1R antagonist significantly attenuated the sleep inducing effects of ethanol[45], suggesting that adenosinergic signaling plays a critical role in mediating the sleep promoting effects of ethanol[46]. Additionally, our current study showed that LL reduced adenosine levels while increasing ethanol drinking. In future studies it will be interesting to determine whether LL-mediated reductions in adenosine levels may cause higher ethanol intake by

reducing the hypnotic effects of ethanol. It is interesting that both total activity and adenosine levels are decreased in LL. Although decreased adenosine in the mice would have increased activity, the decrease in total activity could be due to the masking effects of light and may not be related to changes in adenosine signaling. In nocturnal animals, light inhibits activity and in diurnal animals, light stimulates activity. Given that circadian-related disruptions are associated with augmented alcohol drinking, LL-induced disruption of circadian activity would be expected to increase ethanol intake. In hamsters, LL increased ethanol consumption compared to LD[47], which was consistent with our finding that LL significantly increased ethanol drinking in male mice. However, this finding differs from that of rats, where LL has a suppressive effect on ethanol intake[48]. We postulate that the difference may be due to prior constant dark (DD) exposure, which could suppress the effects of LL on ethanol intake[48].

In general, mice typically become arrhythmic after approximately two weeks exposure to LL[38]. Surprisingly, we found that male ENT1 KO mice appeared to be protected from LL-induced circadian arrhythmicity. However, loss of ENT1 and LL increased ethanol drinking. ENT1 KO mice had lower adenosine levels in the striatum which may underlie the higher ethanol intake[17]. LL also decreased adenosine levels in the NAc which may contribute to higher ethanol intake in this study. It is possible that the effects of LL on adenosine levels and gene expression rhythms in the NAc underlie the increased ethanol consumption, independent of the effects of LL on the suprachiasmatic nucleus (SCN). Light sensitive retinal ganglion cells relay light information to various brain regions through both SCN-dependent and independent mechanisms[49]. Exploring differences in the NAc and SCN at the cellular and molecular levels in mice with a long circadian period compared to arrhythmic mice will be useful to understand the neurophysiological mechanisms underlying the role of the circadian system in modulating ethanol intake in future studies.

We chose dopamine D2 receptor (D2R) gene promoter to express ENT1 based on vector and virus availability for overexpression. Although we are using $A_{2A}R$ promoter driven Cre mice for other experiments, $A_{2A}R$ promotor driven overexpressing system is not well characterized. Thus, we decided to use well-characterized overexpression system. Notably, $A_{2A}R$ is co-expressed with D2R in the NAc and shown to play key roles in striatal neuronal function[50]. Previous in situ hybridization studies reveal that 93% of D2R cells contain $A_{2A}R$ mRNA, and 95% of $A_{2A}R$ cells contain D2R mRNA in striatum[51–53]. Thus, D2R driven overexpression of ENT1 would be appropriate to restore striatal ENT1 function in the overlapping $A_{2A}R$ and D2R neurons in the NAc. Restoration of ENT1 expression in the NAc of ENT1 KO mice reduced the effect of LL on ethanol drinking in ENT1 KO mice, which strongly suggests a direct role for ENT1 expression in the NAc in the effect of light on ethanol consumption. However, we cannot exclude the contributions from other brain regions which are believed to regulate ethanol drinking. Our finding revealed that both LL and loss of ENT1 increase ethanol intake. Interestingly, rescuing ENT1 by viral injection into the NAc appeared to partially reverse the increased ethanol intake in ENT1 KO mice under LL, suggesting that other effects of LL may contribute to the increase in ethanol consumption. Since both LL and lacking of ENT1 independently increased ethanol intake, it is possible that rescuing ENT1 by viral injection into the NAc would partially reverse the increased ethanol intake in ENT1 KO mice under LL condition. Additionally, rescuing

ENT1 in the NAc had no significant effect on circadian activity. Since viral-mediated expression of ENT1 in the NAc did not completely restore ethanol consumption to WT levels, it is not surprising that viral-mediated expression of ENT1 in the NAc did not result in WT-like circadian activity in LL. In addition, the differences in homecage activity rhythms between WT and ENT1 KO mice may be related to differences in adenosine signaling in other brain regions involved in regulating rhythmic locomotor activity, such as the SCN.

In humans, males are known to consume more alcohol than females in general[31,32]. Paradoxically, in rodents, female mice consume more alcohol compared to male littermates[54,55]. Sex differences and ovarian hormones affect sleep, circadian rhythms[56], as well as alcohol intake[57]. Interestingly, the estrous cycle can influence *Per2* expression[58]. Since deficiency of PER2 increases ethanol intake[59], the estrous cycle may influence ethanol drinking through altering *Per2* or other clock genes. In humans, older women with menopause are more vulnerable to AUD[60,61]. Thus, female mice might be more sensitive to stress or hormone-induced changes of ethanol drinking compared to impact of LL. Notably, LL decreased adenosine levels in the NAc only in male mice during the daytime, which might be correlated with the reduction of A_1R and $A_{2A}R$ levels, and were only observed in male, not in female mice. Furthermore, it is also important to confirm whether sex hormones, LL-induced stress, the stages of estrous cycle, x chromosome-specific genetic factors, or developmental effects of gonadal hormones influence ethanol intake and circadian rhythms. Nonetheless, our findings provide a novel sex-dependent role of ENT1 in regulating circadian rhythms and ethanol drinking.

Considering classical perspectives on the interactions between neurotransmitter levels and GPCRs surface expression[62–64], our finding is unexpected. We revealed that adenosine levels were decreased in the striatum, and both A_1 and A_{2A} receptors were also reduced. We have not examined whether A_1R or $A_{2A}R$ are transiently increased to compensate the loss of adenosine levels. However, LL-induced reduction of adenosine levels may also dampen the A_1R and $A_{2A}R$, although additional experiments are required to clarify the impact of LL on adenosine levels and receptors in the future. Since mice lacking ENT1 have reduced adenosine levels in the striatum[17], hypo-adenosine and reduced adenosine A_1 and or A_{2A}Rs-mediated signaling may contribute to LL-dependent excessive ethanol drinking. Previously, we reported that an $A_{2A}R$ antagonist promoted excessive ethanol drinking in WT, but not ENT1 KO mice[65], suggesting that ENT1 regulates ethanol-related behaviors through $A_{2A}R$. In addition, we previously found that activation of adenosine A1R reduced ethanol consumption in ENT1 KO mice[18]. Therefore, the lower level of both A_1R and $A_{2A}R$ observed in ENT1 KO mice, may contribute to the increased ethanol drinking of ENT1 KO mice. A_1R and $A_{2A}R$ are expressed in the different neurons populations in the NAc that have opposing functional roles[66]. A_1R is mainly expressed in the dMSNs while $A_{2A}R$ is expressed in the indirect MSNs (iMSNs). Considering the opposing effects of the two receptors on GPCR signaling (Gi for A_1R and Gs for $A_{2A}R$), it is understandable that downregulation of both A_1R and $A_{2A}R$ could promote ethanol consumption[64].

In conclusion, our study demonstrates that striatal adenosine and ENT1 contribute to the effects of LL on ethanol drinking in male mice, but not female mice. We also revealed that

LL lengthened circadian period in the absence of ENT1, which may correlate with the excessive ethanol intake of ENT1 KO mice. Our study supports a role of ENT1 in maintaining circadian rhythms and regulating ethanol drinking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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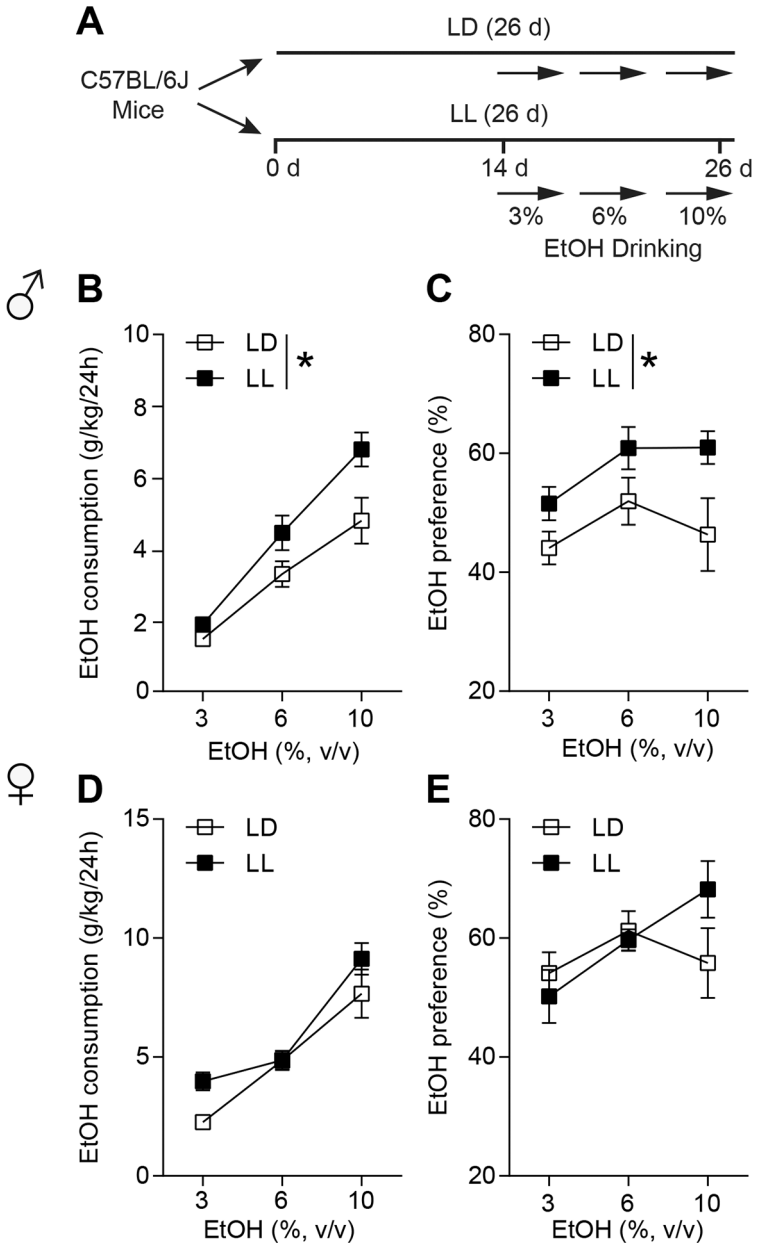

Figure 1.

Constant light (LL) increased ethanol drinking in the male, not female mice. **(A)** The experimental schedule for panels b to e. **(B)** Ethanol consumption per 24 h of 3%, 6%, and 10% EtOH during either light-dark cycle (LD) or LL in the male mice ($n = 9–12$ per group; $*P < 0.05$). **(C)** Ethanol preference per 24 h of 3%, 6%, and 10% EtOH during either light-dark cycle (LD) or LL in the male mice ($n = 9–12$ per group; $*P < 0.05$). **(D)** Ethanol consumption per 24 h of 3%, 6%, and 10% EtOH during either light-dark cycle (LD) or LL in the female mice ($n = 10$ per group). **(E)** Ethanol preference per 24 h of 3%, 6%, and 10% EtOH during either light-dark cycle (LD) or LL in the female mice ($n = 10$ per group).

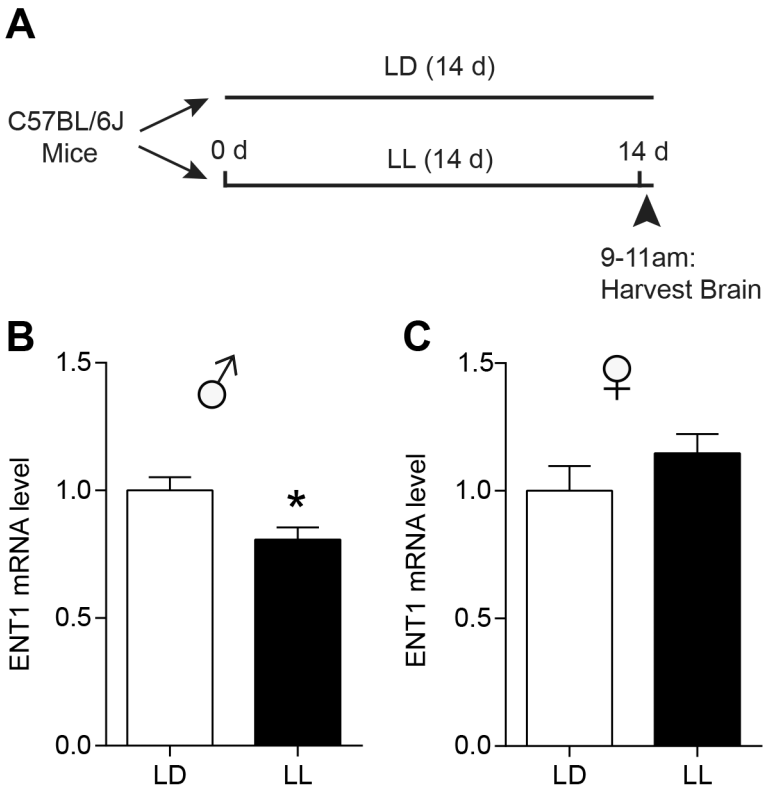

Figure 2.

Constant light (LL) reduced ENT1 mRNA expression in the nucleus accumbens (NAc) of male, not female mice. **(A)** The experimental schedule for panels b and c. **(B)** After exposure of either two weeks of LD or LL, relative mRNA level of ENT1 in the NAc of male mice were examined and shown in the graph ($n = 8$ per group; $*P < 0.05$). **(C)** After exposure of either two weeks of LD or LL, relative mRNA level of ENT1 in the NAc of female mice were shown in the graph ($n = 9$ per group). Data represent mean ± SEM.

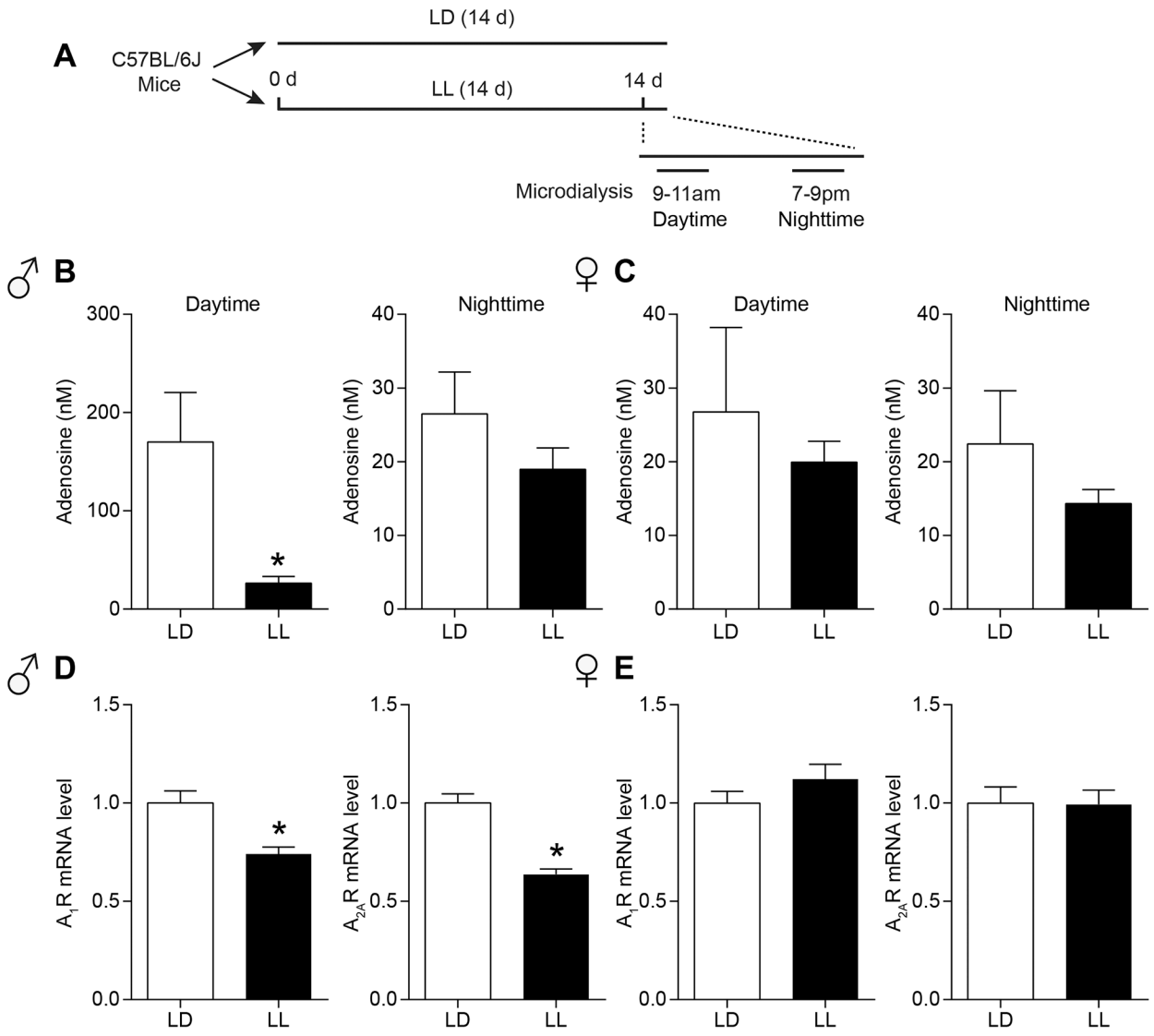

Figure 3.

The effects of constant light on adenosine levels during daytime and nighttime and adenosine receptors in the NAc. **(A)** The experimental schedule of microdialysis for panels b and c. **(B)** Measurement of adenosine levels in the NAc dialysates of male mice during either LD or LL at daytime (n = 10–12 per group; *P < 0.05) or nighttime (n = 8–9 per group). **(C)** Measurement of adenosine levels in the NAc dialysates of female mice during either LD or LL at daytime (n = 8–10 per group) or nighttime (n = 7–8 per group). **(D)** After exposure of either two weeks of LD or LL, relative mRNA level of A_1R and $A_{2A}R$ in male mice were detected and shown in the graph (n = 9 per group; *P < 0.05). **(E)** After exposure of either two weeks of LD or LL, relative mRNA level of A_1R and $A_{2A}R$ in female mice were detected and shown in the graph (n = 9 per group). Data represent mean ± SEM.

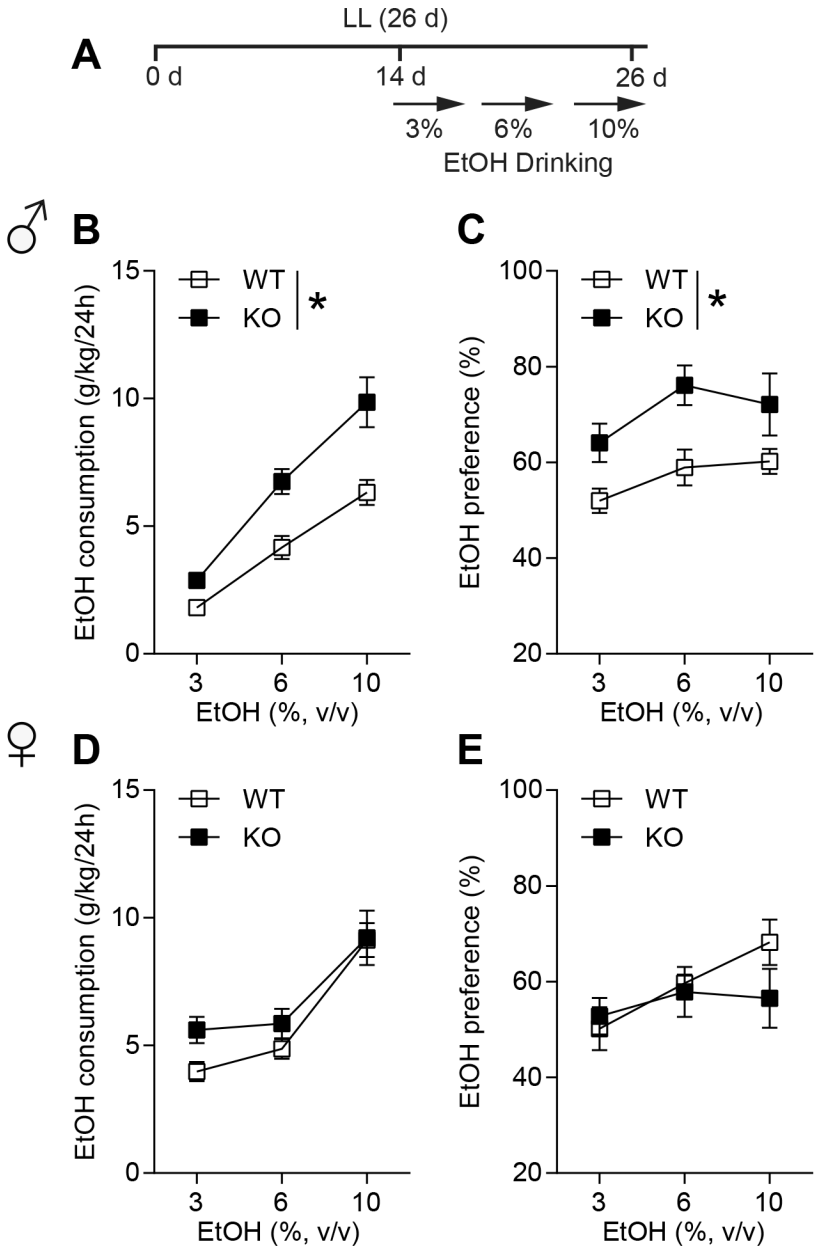

Figure 4.

Male ENT1 KO mice showed increased ethanol drinking not in female. **(A)** The experimental schedule for panels b to e. **(B)** Ethanol consumption per 24 h of 3%, 6%, and 10% EtOH in male WT and ENT1 KO mice during LL (n = 10–11 per group; *P < 0.05). **(C)** Ethanol preference per 24 h of 3%, 6%, and 10% EtOH in male WT and ENT1 KO mice during LL (n = 10–11 per group; *P < 0.05). **(D)** Ethanol consumption per 24 h of 3%, 6%, and 10% EtOH in female WT and ENT1 KO mice during LL (n = 10 per group). **(E)** Ethanol preference per 24 h of 3%, 6%, and 10% EtOH in female WT and ENT1 KO mice during LL (n = 10 per group). Data represent mean ± SEM.

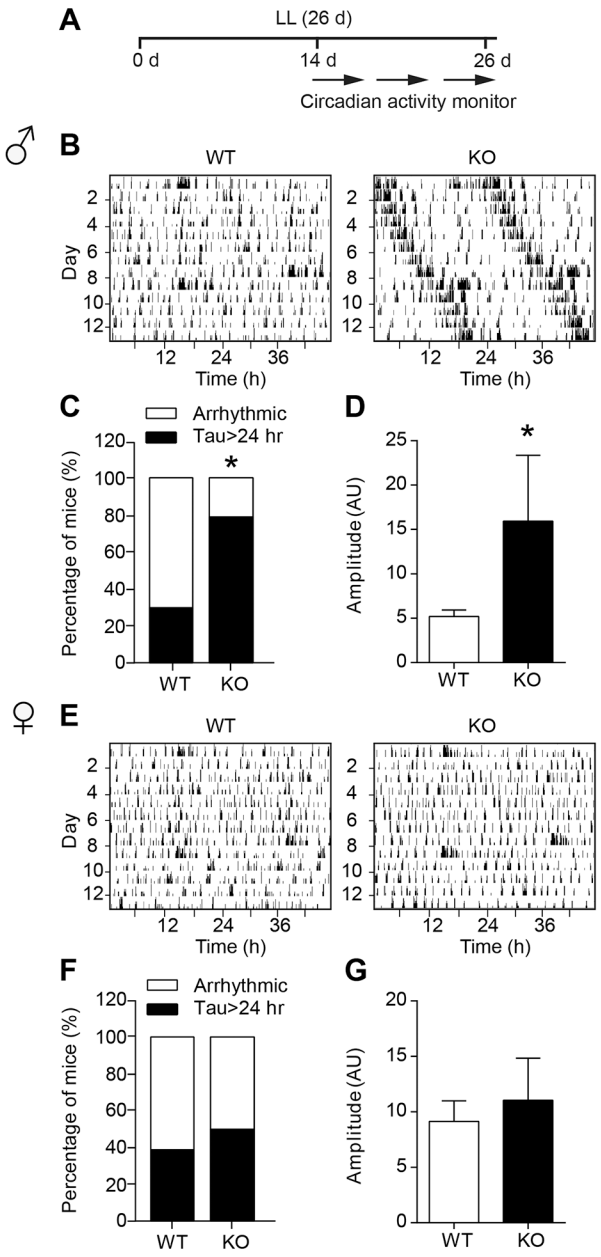

Figure 5.

Male ENT1 KO mice showed longer period and amplitude of circadian activity during constant light not female. **(A)** The experimental schedule for panels b to g. **(B)** The representative image for circadian activity pattern of male WT and KO mice during LL. **(C)** The percentage of mice number in male WT or ENT1 KO group showing either clear peak or arrhythmic during LL ($n = 10$ per group; $^*P < 0.05$). **(D)** The amplitude of male WT and KO mice during LL ($n = 10$ per group; $^*P < 0.05$). **(E)** The representative image for circadian activity pattern of female WT and KO mice during LL. **(F)** The percentage of mice number in female WT or ENT1 KO group of showing either clear peak or arrhythmic during LL ($n = 10$ per group). **(G)** The amplitude of female WT and KO mice during LL ($n = 10$ per group). Amplitude was measured by arbitrary unit (AU). Data represent mean ± SEM.

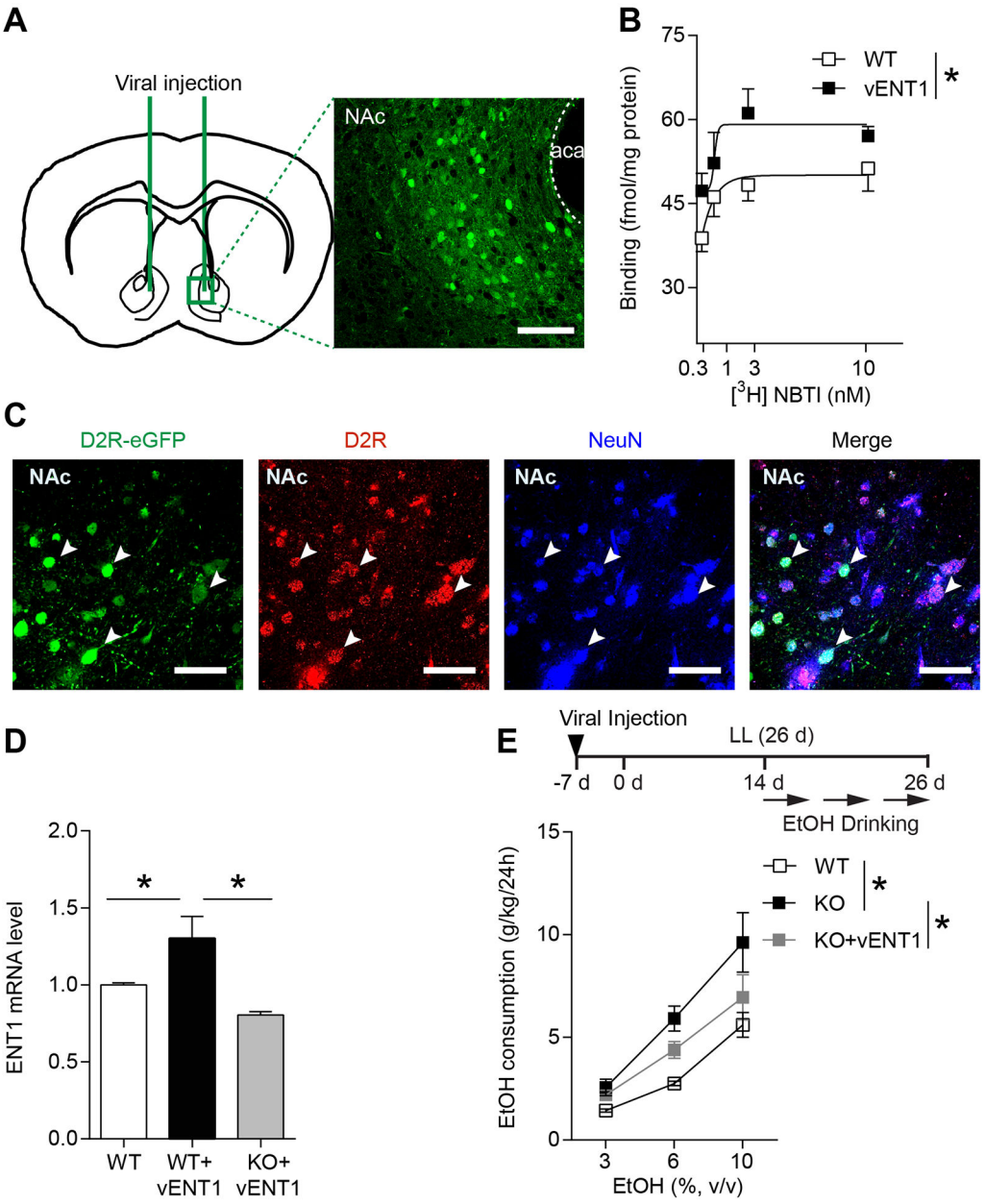

Figure 6.

Restoring accumbens ENT1 suppressed the high ethanol drinking in ENT1 KO mice during constant light. **(A)** The location of viral injection into the NAc of ENT1 KO/WT mice. Representative fluorescence image was shown at x20 magnification. Scale bar=100 μm. **(B)** Binding of [^{3}H] NBTI to the NAc membranes, showing increased binding sites in ENT1 virus injected WT mice (vENT1) compared to control (WT) group ($n = 6$ per group; $^*P < 0.05$). **(C)** The confocal images showing co-localization of eGFP virus, D2R, and NeuN (white arrows) indicated that D2R-eGFP virus was specifically expressed in D2R-positive neurons. Scale bar=50 μm. **(D)** Relative mRNA level of ENT1 in the NAc of WT mice, ENT1 virus injected into WT mice (WT+vENT1), and ENT1 virus injected into ENT1 KO mice (KO+vENT1; $n = 5–6$ per group; $^*P < 0.05$). **(E)** Ethanol consumption per 24 h of 3%,

6%, and 10% EtOH in WT, KO, and KO+vENT1 group during LL ($n = 5$ per group; $*P < 0.05$). Data represent mean ± SEM.