The Effect of Prefrontal Cortex Specific Knockout of *Bmal1* on Mood-Like and Alcohol Binge Drinking Behavior in Male and Female Mice

Nour Qutеishat

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By: Nour Quteishat

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Signed by the final examining committee:

_____ Chair

Andreas Arvanitogiannis

_____ Examiner

Andrew Chapman

_____ Examiner

Richard Courtemanche

_____ Thesis Supervisor(s)

Shimon Amir

_____ Thesis Supervisor(s)

Uri Shalev

Approved by:

_____

Chair of Department, Andrew Ryder

_____ 2024 _____

Dean of Faculty, Pascale Sicotte

ABSTRACT

The effect of Prefrontal cortex specific knockout of *Bmal1* on mood-like and alcohol binge drinking behavior in male and female mice

Nour Qutеishat

The circadian rhythm, regulated by clock genes, plays a pivotal role in regulating physiological and behavioral processes, including mood regulation and alcohol consumption. Despite significant advancements in understanding the interplay between circadian dysregulation and affective disorders, the specific role of *Bmal1* within the prefrontal cortex on modulating mood-like behaviors and alcohol binge drinking in not fully understood. This research aims to address this gap by investigating the effects of selectively deleting *Bmal1* in the prefrontal cortex on these behavioral outcomes in male and female mice. Female *Bmal1* knockout mice exhibited reduced depressive-like behaviors and heightened anxiety-like behaviors. This contrasted with male counterparts where no significant alterations in mood-related behaviors were observed. This study provides new perspectives into the role of *Bmal1* in the prefrontal cortex in affective behavior and alcohol binge drinking male and female mice, emphasizing the importance of considering sex-specific responses.

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CONTRIBUTION OF AUTHORS

Dr. Shimon Amir, Dr. Konrad Schöttner and Nour Quteishat collaborated to design the experiments. Nour Quteishat performed surgeries and conducted behavioral experiments. Arianne Menasce aided in the ethanol binge-drinking experiments. Nour Quteishat performed data analysis and visualization. The manuscript was primarily authored by Nour Quteishat, with input and editing from Dr. Shimon Amir and Dr. Konrad Schöttner.

All authors reviewed the final manuscript and approved all the contents.

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Affective disorders, such as mood and anxiety disorders, are prevalent mental illnesses that significantly impact the daily lives of those affected. In Canada, mood disorders affect around 5.4% of the population (Leclerc et al., 2020), while substance use disorders affect approximately 21% (Jim Moore, 2021). Affective disorders are strongly comorbid with substance-use disorders, with individuals experiencing mood disorders being twice as prone to substance abuse compared to the general population (Rush et al., 2008). Previous research has focused on exploring the neural mechanisms involved in these conditions. One notable similarity among these illnesses is the disruption of daily rhythms, such as an altered sleep-wake cycle, suggesting that circadian dysregulation plays a role in affected patients (Logan & McClung, 2019). Studies have also identified genetic associations in humans, linking core clock genes to alcohol consumption and an increased risk of alcohol use disorders (Davis et al., 2018; Partonen, 2015), as well as associations between circadian genes and major depressive disorders (Christiansen & Bouzinova, 2017). Therefore, understanding the involvement of clock genes in mood and alcohol use disorders is crucial for enhancing treatment and diagnosis approaches.

The circadian system in mammals is a complex network of biological pacemakers, with the master circadian clock located in the suprachiasmatic nucleus (SCN). Its main role is to generate daily rhythms of physiological and behavioral processes aligned to the external 24-hour external environment. This is important so that these processes happen at the appropriate time of the day/night. These internal rhythms are generated through the interplay of a set of clock genes and their protein products, which establish interconnected feedback loops. This process involves the orchestration of daily patterns of MRNA transcription and translation into proteins, as well as accumulation, and degradation of the clock proteins thereafter. At the molecular level, the primary transcription-translation feedback loop (TTFL) includes Brain and Muscle Arnt-Like 1 (*Bmal1*), Circadian Locomotor receptor Output Cycles Kaput (Clock), *Period* (*Per 1-3*), and *Cryptochrome* (*Cry 1-2*) (Takahashi, 2017). *Bmal1* and CLOCK form a positive loop that activates the transcription of *Per* and *Cry* by specifically binding to E-boxe elements in their respective promoters (DeAssis & Oster, 2021; Takahashi, 2017). In turn, PER and CRY dimerize to form a complex that inhibits the transcriptional activity of BMAL1/CLOCK, thereby repressing their own transcription and establishing a negative feedback loop. The degradation of PER and CRY allows the cycle of transcription and translation to initiate once more. Clock

genes play a critical role in the intricate mechanism of the molecular clock. Consequently, disruptions in the expression of these clock genes can have a profound impact on cellular processes, potentially contributing to the development of pathological conditions (Ketchesin et al., 2018).

Apart from the central clock located in the SCN, there are peripheral clocks found in various brain regions (Barclay et al., 2012), one specific area being the prefrontal cortex (PFC). The PFC can be further subdivided into two distinct regions known as the lateral and ventromedial PFC (Striedter, 2005). Among these, the medial prefrontal cortex (mPFC) is involved in higher-order executive processes and projects to brain regions that influence mood, motivation, and impulsivity (Klenowski, 2018).

The PFC exhibits rhythmic expression of clock genes (Christiansen et al., 2016), which has a significant impact on its function. For example, clock genes influence the regulation of dopamine circuitry in the PFC (Verwey et a., 2016). Additionally, the release of glutamate and gamma-aminobutyric acid (GABA) within the PFC is under clock gene control (Roberts & Karatsoreos, 2023; Saitoh et al., 2014; Hasler et al., 2007). Conversely, dopamine release affects the expression of clock genes in the PFC (Kim et al., 2017). This indicates a reciprocal relationship between circadian clock gene expression and dopamine signaling, which could potentially play a role in regulating affective disorders and substance abuse.

Studies on humans and rodents have linked molecular clock genes to the development of mood and substance use disorders. Postmortem human brain samples have indicated significant alterations in circadian clock genes within the PFC among individuals diagnosed with major depression disorder (MDD) (Li et al. 2013; Francis & Pocru, 2023). Similarly, analyses of suicide victims have shown a decrease in clock-controlled genes within the PFC (Sequeria at al., 2012; Christiansen & Bouzinova, 2017). Additionally, postmortem studies have established a connection between depression and reduced neuroplasticity in the PFC, which is closely linked to altered chronobiology (Duman & Voleti, 2012; Eyre & Baune, 2012). Furthermore, previous research has indicated an association between polymorphism in the human *Bmal1* and *Per2* genes and alcohol consumption and abuse (Partonen 2015), and polymorphism in human *Clock*, *Bmal1* and *Per2* is associated with affective disorders (Kripke et al., 2009). Therefore, all the evidence points to a critical role of circadian clock genes in the PFC in the regulation of addiction, mood and affective disorders.

Research on rodent models further highlights the significance of clock gene expression in the PFC on depressive- like behaviors and alcohol consumption. Mice with induced negative mood phenotypes displayed disrupted rhythms of *Per1*, *Per2*, *Rev-erba* and *Bmal1* in the PFC

(Otsuka et al., 2020). Mice with depressive-like behavior induced by chronic mild stress (CMS), exhibited a reduction in *Bmal 1* and *Clock* gene levels in the PFC (Calabrese et al., 2016). Additionally, rats subjected to simulated night shift work showed reduced *Bmal1* phosphorylation in the PFC, which could lead to an elevated risk of affective-like behaviors (Marti et al., 2017; Zheng et al., 2023). Regarding alcohol consumption, Melendez el al. (2011) reported significant changes in gene expression within the PFC following chronic intermittent exposure to alcohol. Similarly, Del Olmo et al. (2019) reported altered expression of *Clock*, *Per2* and *Bmal1* in the PFC following ethanol binge drinking and high-fat diet consumption. These findings indicated an association between clock genes in the PFC, mood, and alcohol drinking behaviors.

Previous research indicates a connection between disruptions in circadian clock genes and depression and alcohol-use disorders. Evidence suggests that changes in clock gene expression within the PFC may contribute to these disorders, possibly through the interactions with dopamine signaling (DePoy et al., 2017; Hare & Duman. 2020). The purpose of this study is to specifically investigate the impact of *Bmal1* deletion in the PFC on depressive-like behavior and alcohol binge drinking. We hypothesized that altering the expression of this clock gene within the PFC would lead to changes in behaviors associated with depression and alcohol binge drinking.

Methods

Animals

This study utilized male and female transgenic mice, 12 to 16 weeks of age, carrying floxed alleles of the *Bmal1* gene (*Bmal1*$^{fl/fl}$; B6.129S4(Cg)-Arntltm1Weit/J; stock number: 007668, Jackson Laboratory). All animals were group-housed, two to four subjects per cage, on a 12 h light/ 12 h dark cycle, with food and water provided *ad libitum*. Room temperature and relative humidity was maintained at 21 $\pm$ 1 ℃ and 65 $\pm$ 5%, respectively. Cages were changed once a week. After stereotactic surgery was performed, animals were individually housed in light- and soundproof boxes under similar conditions. All animal procedures were conducted in accordance with the Animal Care Committee of Concordia University (certificate number: 30000256) and approved by the Canadian Council on Animal Care.

Stereotactic surgery

All mice underwent a bilateral stereotactic injection of an Adeno Associate Virus (AAV) vectors expressing either Cre-Recombinase and Enhanced Green Fluorescence Protein (EGFP) (AAV2/5-CAG-CRE-EGFP, 1.0 x 10^{12} vg/ml, Molecular Tool Platform, Quebec,

Canada) or EGFP only (AVV2/5-CAG-EGFP, 1.0 x 10^{12} vg/ml, Molecular Tool Platform, Quebec, Canada), administered in the Prefrontal Cortex (PFC). The former will be referred to as KO and the latter as WT. The surgical procedure was performed using a Somnosuite machine (Kent Scientific Corporation, Torrington, CT, USA) with 2% isoflurane anesthesia. The surgery was conducted at specific coordinates targeting the PFC – anteroposterior: 1.78 mm anterior to bregma, mediolateral: $\pm$0.1 mm lateral to midline, dorsoventral: -2.0 mm ventral to the skull (taken as "0") (Paxino & Franklin, 2001). A volume of 200 nl of the viral vector was injected at a rate of 100 nl/min. Mice received postoperative analgesic ketoprofen (5 mg/g; Merial), both immediately after surgery and every 24 hours over a total period of 48 hours. All behavioral procedures were conducted three weeks after the delivery of the viral vector.

Behavioral Procedures

Various behavioral tests were conducted to assess anxiety and depressive- like behaviors, as well as binge drinking behavior in mice. The Sucrose Preference test (SPT), Open Field Test (OFT), Novelty Suppressed Feeding (NSF), and Tail Suspension Test (TST) were used to evaluate anxiety- and depressive-like behaviors with at least 4 days interval between tests. The drinking in the dark (DID) test was used to assess binge drinking behavior. All behavioral tests were conducted between ZT 2-6 (i.e., 2-6 hours after lights-on), except for the drinking in the dark test at ZT 15, and the sucrose preference test conducted at ZT 2 and ZT 14. The mice were habituated to the testing environment for an hour before starting the experiment. Body weights were recorded after each behavioral test.

Measures

Sucrose Preference Test

The Sucrose Preference Test was used as an assay to evaluate hedonic state and anhedonia. A reduction in sucrose intake compared to water intake is indicative of anhedonia, which can be a sign of depressive- like behavior in rodents (Liu et al. 2018). Three days prior to the experiment, the mice were habituated to having access to two drinking bottles for 48 hours. One bottle contained tap water, while the other contained a solution of 1% sucrose in water. On the third day, the mice had access to two water bottles. On the fourth day, the mice were given access to one bottle of water and one bottle of 1% sucrose for three hours at ZT2. After a day of rest with access to two water bottles, the mice were again given access to one bottle of water and one bottle of 1% sucrose for three hours at ZT 14. The positions of the bottles were switched for each test to reduce any potential confounds produced by a side bias. Water and sucrose consumption were measured by weighing the bottles before and after the tests. The

percentage of sucrose preference was determined by comparing the consumed percentage of sucrose solution to the percentage of water consumed.

Open Field Test

The open field test was used to evaluate anxiety- like behavior. This is based on the natural aversion of mice to open and unknown environments (Gould et al., 2009). After habituation, animals are placed in the open field arena (45 cm x 45 cm x 40 cm) equipped with infrared beams and surrounded by plexiglass walls. The subject's activity is recorded for 10 minutes using the Actitrack software package (Panlab, Barcelona, Spain). Several behavioral parameters were measured, including total distance traveled, time spent in the center of the arena, and the latency to enter the center of the arena.

Novelty Suppressed Feeding

The novelty suppressed feeding test was used to measure depressive- like behavior. The test is based on the natural aversion of rodents to brightly lit and open spaces which represents a stressful and novel environment (Stedenfeld et al., 2011). A longer latency to start feeding indicated decreased motivation or anhedonia. The mice were subjected to food deprivation for 24 hours before the test. Initial weights of the subjects were recorded prior to the test commencement. During the test, the subject was placed in a brightly lit arena (61 cm x 61 cm x 50 cm) with a pellet of fresh food placed in the center while video was recorded (Samsung Galaxy A5 2017 phone). The subject was allowed to explore the arena for 12 minutes or until it took a bite of the food. The time taken by the subject to begin feeding, recorded in seconds, was noted for each individual. Following the test, the subject was returned to its home cage and allowed free access to a pre weighed food pellet for 8 minutes. This post-test food consumption, in conjunction with the total weight loss measured by comparing the body weight prior to testing, was used as a control mechanism. The aim was to verify that any variations in feeding behavior observed during the test were not due to differences in food intake or body weight.

Tail Suspension Test

The tail suspension test was used to assess depressive-like behavior in mice. The aim of the test is to measure the time the mouse spends immobile, which is considered an indicator of depressive-like behavior (Cryan et al., 2005). The mice were suspended by the tip of the tail to a metal bar that was 30 cm above ground, using adhesive tape. A plastic tubing was placed above the tail as a safety measure to prevent the animal from climbing or injuring itself during the test. The total duration of the test was 6 minutes, during which time video was recorded (Samsung Galaxy A5 2017 phone). The mobility time of the mouse was analyzed using Stopwatch+ software (Center for Behavioral Neuroscience, Georgia State University, Atlanta,

GA, USA). The immobility time was then calculated by subtracting the mobility time from the total experiment time.

Drinking in the dark test

The 4-day drinking in the dark test was used to measure binge drinking behavior (Thiele et al., 2014). During the experiment, mice were given access to a bottle of 20% ethanol solution (v/v) in tap water three hours into the dark cycle (ZT15). This access occurred for two hours on the first three days and four hours on the fourth day. This 4-day paradigm is conducted twice over a two-week period. The measurement of ethanol solution consumption was achieved by calculating the difference in ethanol levels within the bottles before and after the test. During the fourth session of the experiment, which spanned 4 hours, ethanol consumption was measured at two-hour intervals. Quantification occurred at both the 2-hour and 4-hour marks. Statistical analysis was conducted using a 2-way repeated measure analysis of variance (RM-ANOVA), considering session (i.e., 8 sessions) and virus (i.e., KO vs WT). A separate 2-way ANOVA was also conducted on the fourth and eighth day of the session, considering time (per 2 hours) and virus.

Viral Spread Validation

Following dissection, the brains of all subjects were flash-frozen and stored at -80℃. Subsequently, these brains were sliced on a Cryostat to validate regional specificity of the viral delivery and the extent of the viral spread under a fluorescence microscope. Animals that exhibited improper viral distribution were excluded from the experiment. Afterward, images detailing the viral distribution and specificity were inputted into Inkscape software (Inkscape Project, 2020) and combined to produce a composite that represented the average viral spread and delivery.

A subset of brains was collected to conduct immunofluorescence staining. For this, transcranial perfusion was employed. This process involved circulating a solution of cold saline (0.9% NaCl) and paraformaldehyde (PFA, 4% in 0.1M phosphate buffer, pH 7.3) through the mouse's blood vessels, ensuring tissue preservation. Following a 24-hour post-fixation period (in 4% PFA at 4℃), brains were immersed in a 30% sucrose solution for 48 hours. Subsequently, the brains were then embedded in cryomatrix (Thermo Fisher Scientific, Western Michigan University, Kalamazoo, MI, USA) and stored at -80℃. This preservation method was employed to prevent the separation of brain slices during the slicing process in the Cryostat at 20μm. The brain sections were used for future immunofluorescence imaging.

Immunofluorescence

Brains previously embedded in cryomatrix were sliced in the Cryostat at 20μm and affixed onto microscope slides. The process of immunofluorescence was conducted directly on these slides, with the utilization of a hydrophobic pen to create a square barrier around each brain slice. Initially, the brain slices were rinsed in phosphate buffered saline (PBS, pH 7.4) for 10 minutes. This was followed by rinsing the sections three times in 0.3% Triton-X in PBS (PBS-Tx) for 10 minutes each time. Subsequently, the sections were immersed in a blocking solution (3% milk powder in PBS-Tx, 6% Normal Donkey Serum) for one hour at room temperature. Following this, the sections were subjected to immunofluorescent staining using two different antibodies: a monoclonal Anti-Cre antibody produced in mice (1:2000, C7988, Sigma-Aldrich) and a polyclonal Anti-Bmal1 antibody produced in rabbits (1:500, NB100-2288, NovusBiologicals). After another set of three 10-minute rinses with PBS-Tx, the sections were subjected to immunofluorescent staining with two secondary antibodies: Alexa594 produced in mice (1:500, A-21203, Thermo Fisher) and Alexa647 (1:500, 711-605-152, Jackson) produced in rabbits. Following a final rinse with PBS-Tx, the slides were coverslipped and stored in a refrigerator. Finally, images were captured using confocal microscopy (Olympus Fluoview FV10i), and representative images were generated using Image J (Imagej Software, 2021) and Inkscape software (Inkscape Project, 2020).

Statistical Analysis

Prism 10 (GraphPad Software, San Diego, CA, USA) was used to conduct all statistical analysis. The values are reported as means $\pm$ standard error of mean (SEM). For between-group comparisons of behavioral tests, unpaired two-tailed t-tests were used. For ethanol-drinking procedures, two-way repeated measures ANOVA (virus by session) was used, followed by post-hoc analysis of significant main effects. The null hypothesis of no model effects was rejected at $p < 0.05$.

Results

Validation of prefrontal cortex specific *Bmal1* knockout

The effectiveness and specificity of prefrontal cortex (PFC) knockout were confirmed using fluorescence microscopy (Fig. 1) and through immunofluorescence staining (Fig. 2). Analysis of images containing DAPI (Fig. 2B), Green Fluorescent Protein (GFP; Fig. 2C), neuronal nuclei (NeuN; Fig. 2D), and *Bmal1* (Fig. 2E) indicated that cells within the PFC infected with GFP-expressing virus did not show *Bmal1* expression and vice versa.

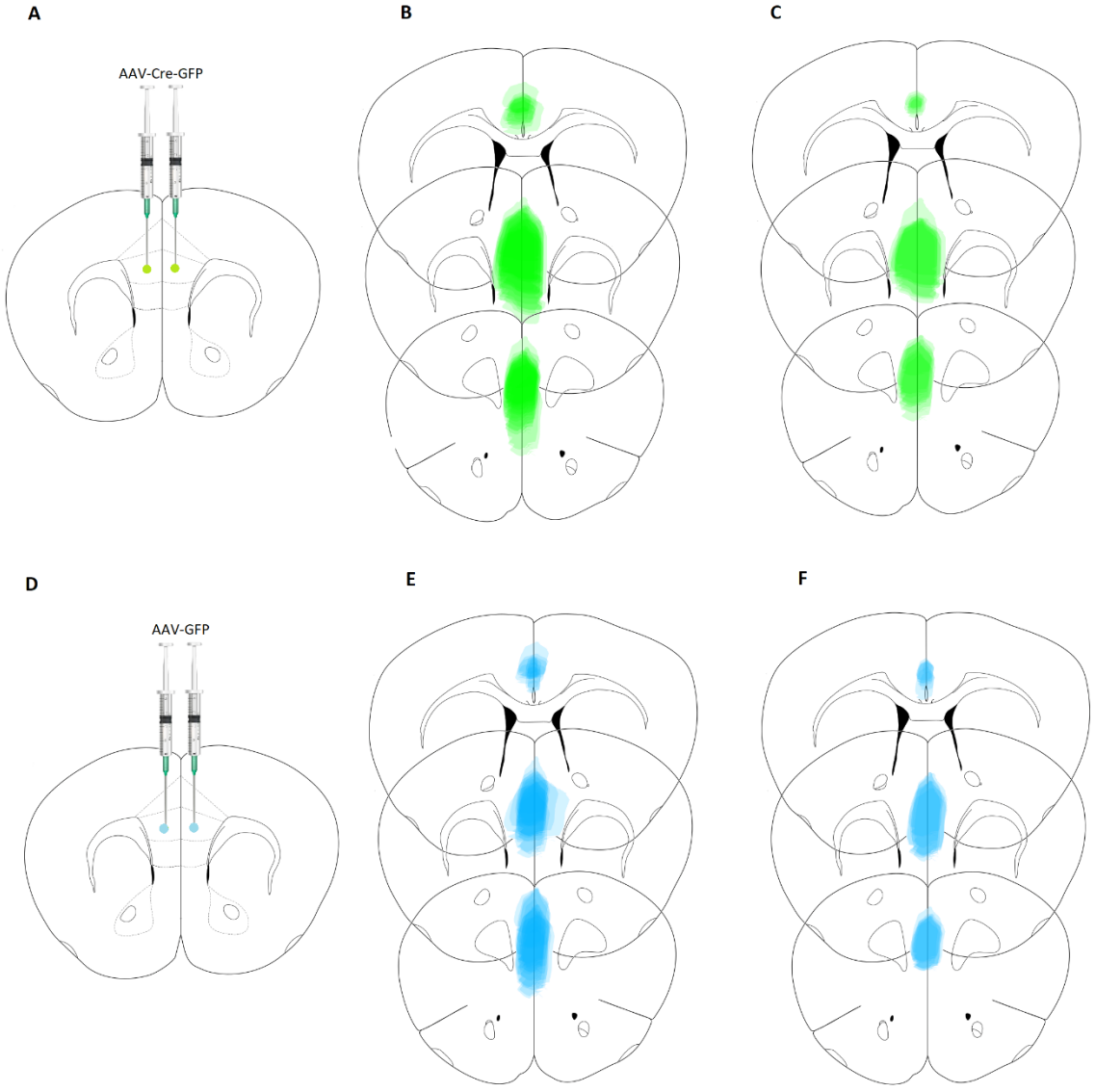

Figure 1. Illustrative images of viral *Bmal1* knockout validation under Fluorescence microscopy
The schematic representation illustrates the administration of AAV-Cre-GFP (A) and AAV-GFP (D) within the PFC. Additionally, schematic diagrams are presented in an anterior-to-posterior sequence, showcasing the confirmed *Bmal1* knockout overlap in males (B) and females (C), alongside control subjects in males (E) and females (F).

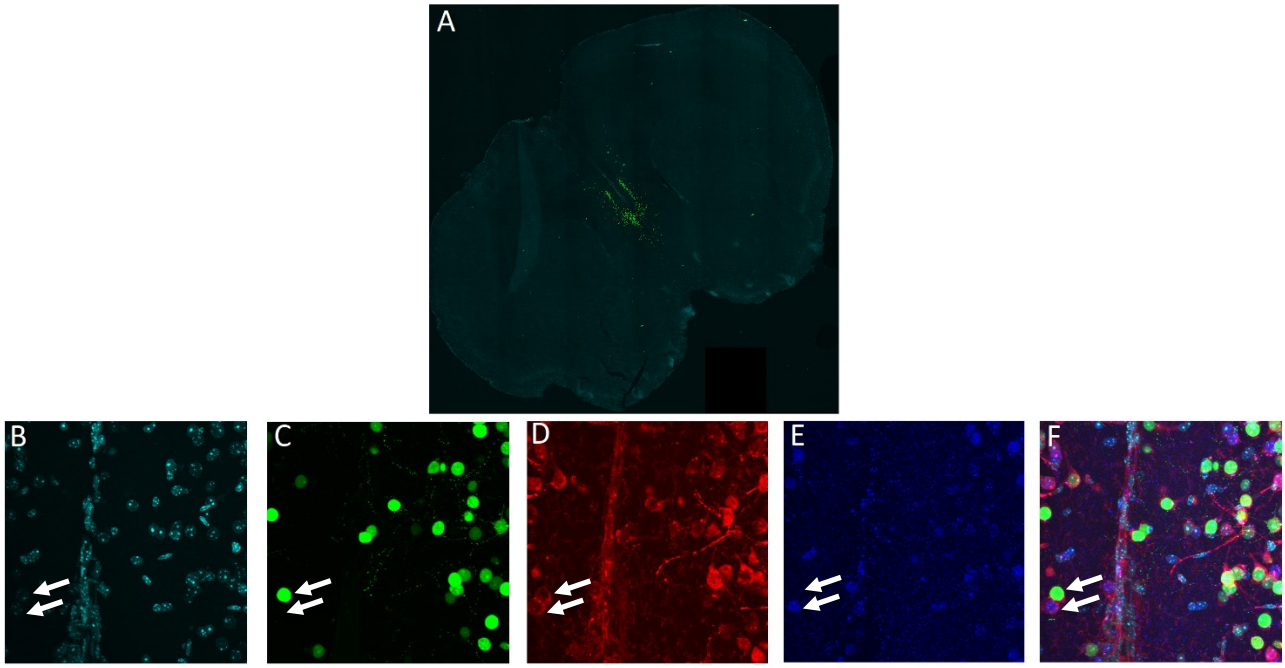

Figure 2. Validation of *Bmal1* prefrontal cortex viral knockout
Immunofluorescence staining images display coronal sections demonstrating specific virus expression within the PFC (A). Representative images of labeled cells in the PFC of *Bmal1* knockouts are presented as follows: DAPI in cyan (B), GFP in green (C), *Bmal1* in blue (E), and a merged representation (F). Arrows indicate a cell infected with virus in junction with an uninfected one.

Depressive- like behavior of PFC specific *Bmal1* knockout

To examine the impact of *Bmal1* deficiency in the prefrontal cortex on depressive-like behaviors, male and female *Bmal1* knockouts and control group mice were evaluated using three behavioral tests: the Sucrose Preference Test (SPT) (Fig. 3), Novelty Suppressed Feeding test (NSF) (Fig. 4), and Tail Suspension Test (TST) (Fig. 5).

Bmal1 knockout in the PFC did not have an impact on depressive-like behaviors in male mice, regardless of the specific behavioral tests conducted (Fig. 3-5). Comprehensive statistical analysis results can be found in Table 1.

In female mice, *Bmal1* deletion in the PFC reduced depressive- like behavior in the SPT (Fig. 3). Specifically, female *Bmal1* knockouts had an 11% increased preference for sucrose solution at ZT 2 compared to controls. This difference was not observed at ZT 14. In addition, female controls displayed an increased preference for sucrose solution at ZT 14 compared to ZT 2 (Fig. 3) which was approaching significance. This time of day- dependent difference in sucrose preference was not observed in the female knockout animals. Lastly, *Bmal1* knockouts in females did not affect depressive-like behavior in the NSF or the TST (Fig. 4-5)

Anxiety- like behavior of PFC specific *Bmal1* knockout

To investigate the impact of *Bmal1* knockout in the PFC on anxiety- like behaviors, the open field test (OFT) was performed (Fig. 6). No significant differences were observed between male *Bmal1* knockout or female *Bmal1* knockout and their respective control groups in the total distance traveled and percent time spent in the center of the field. In contrast, female *Bmal1* knockouts exhibited a significant difference from controls in terms of the latency to enter the center of the field. More specifically, female knockouts, on average, displayed a 10-second delay in entering the center of the open field when compared to controls.

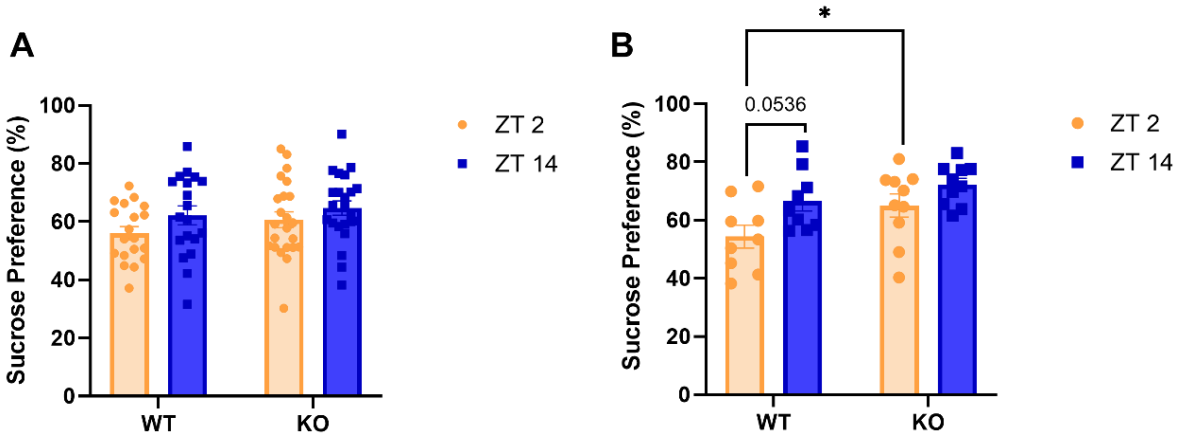

Figure 3. Depressive- like behavior of prefrontal cortex specific *Bmal1* knockouts in the sucrose preference test. The sucrose preference test was used to assess the effect of *Bmal1* knockout in the PFC on depressive- like behavior in male (A, *n* = 19-23) and female mice (B, *n* = 9-10) at two different time points (ZT 2 & ZT 14). No significant difference in sucrose preference was observed between male *Bmal1* knockout and controls at either time point. In contrast, female *Bmal1* knockout mice displayed an increased preference for sucrose compared to controls, but only at ZT 2. Additionally, the sucrose preference of female control mice varied between the two time points, with an increase at ZT 14. This temporal difference was not observed in the *Bmal1* knockout group. The results are presented as mean ± standard error of the mean, with statistically significant difference denoted by * ($p < 0.05$). Further statistical details can be found in Table 2.

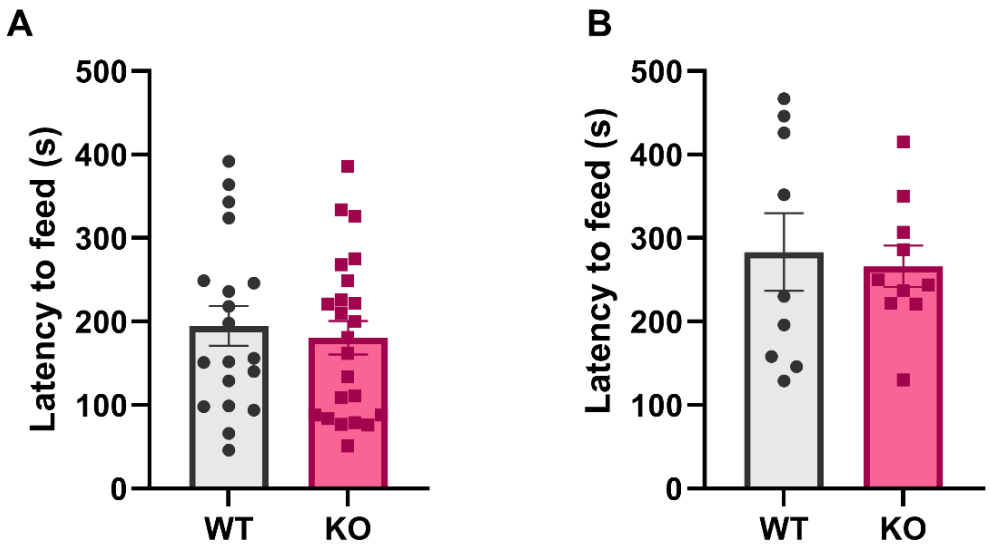

Figure 4. Depressive- like behavior of prefrontal cortex specific *Bmal1* knockouts in the novelty suppressed feeding.

The novelty suppressed feeding test was used to assess the effect of *Bmal1* knockout in the PFC on depressive-like behavior in male (A, n = 19-23) and female mice (B, n = 9-10). No significant difference in the latency to feed was observed between male or female *Bmal1* knockouts and their respective control groups. The results are presented as mean ± standard error of the mean, with statistically significant difference denoted by * ($p < 0.05$). Further details of independent measures t-test can be found in Table 1.

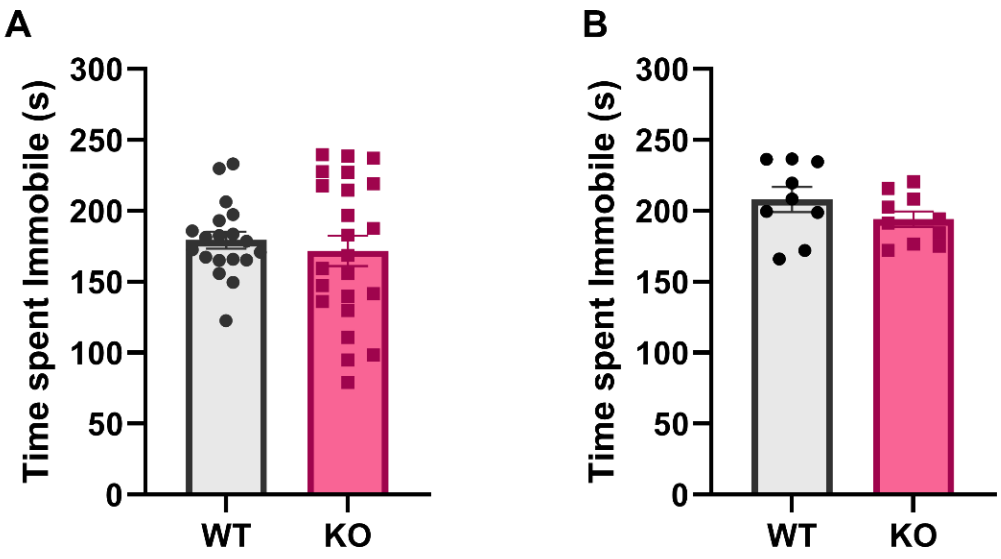

Figure 5. Depressive- like behavior of prefrontal cortex specific *Bmal1* knockouts in the tail suspension test. The tail suspension test was used to assess the effect of *Bmal1* knockout in the PFC on depressive-like behavior in male (A, n = 19-23) and female mice (B, n = 9-10). No significant difference in the time spent immobile was observed between male or female *Bmal1* knockouts and their respective control groups. The results are presented as mean ± standard error of the mean, with statistically significant difference denoted by * ($p < 0.05$). Further details of independent measures t-test can be found in Table 1.

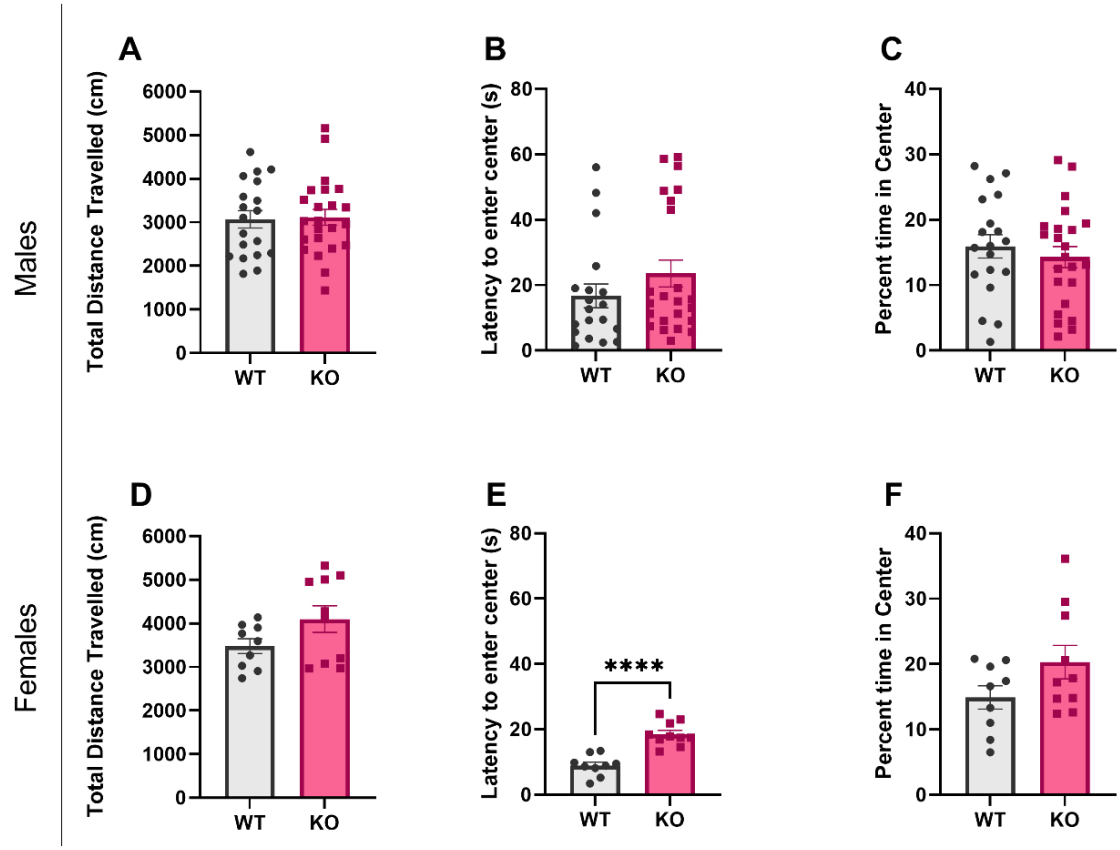

Figure 6. Anxiety- like behavior of prefrontal cortex specific *Bmal1* knockouts in the open field test.
The open field test was used to assess the effect of *Bmal1* knockout in the PFC on anxiety- like behavior in male (A-C, *n* = 19-23) and female mice (D-F, *n* = 9-10). No significant difference in total distance traveled, latency to enter center, and percent time in center were observed between male *Bmal1* knockouts and their controls. In contrast, female *Bmal1* knockout mice (E) had an increased latency to enter the center of the open field compared to the control group. Total distance traveled and percent time in center were not affected (D and F). The results are presented as mean ± standard error of the mean, with statistically significant difference denoted by **** ($p < 0.0001$). Further details of independent measures t-test can be found in Table 1.

Table 1. *Behavioral tests in male and female mice with Bmal1 knockout in the PFC*

Behavioral test	Males	Females
	KO vs WT	**KO vs WT**
NSF Latency to feed	$t(40) = 0.456$; $p = 0.651$; $\eta^2 = 0.005$	$t(17) = 0.336$; $p = 0.741$; $\eta^2 = 0.007$
TST % time immobile	$t(40) = 0.589$; $p = 0.559$; $\eta^2 = 0.009$	$t(17) = 1.365$; $p = 0.190$; $\eta^2 = 0.099$
OFT Distance traveled	$t(40) = 0.178$; $p = 0.860$; $\eta^2 < 0.001$	$t(17) = 1.708$; $p = 0.106$; $\eta^2 = 0.147$
OFT Latency to enter center	$t(40) = 1.220$; $p = 0.230$; $\eta^2 = 0.036$	$t(17) = 6.101$; ***p < 0.0001***; $\eta^2 = 0.687$
OFT % time in center	$t(40) = 0.687$; $p = 0.496$; $\eta^2 = 0.012$	$t(17) = 1.699$; $p = 0.108$; $\eta^2 = 0.145$

Note. Statistical calculations performed using alpha level of .05. Significant results are indicated by bolded p-values.

Binge- drinking behavior of PFC specific *Bmal1* knockout

To evaluate the influence of *Bmal1* knockout in the PFC on alcohol binge drinking in male and female mice, a 2-way repeated measure analysis of variance (RM-ANOVA) was performed. Detailed statistical data can be found in Table 2 and 3. The RM-ANOVA revealed no significant difference between the *Bmal1* knockout and control group in male and female mice (Fig. 7).

On the fourth session of the experiment ethanol consumption was measured at two-hour intervals. In the case of male *Bmal1* knockouts, there was no notable difference between the first 2 hours and the last 2 hours on the fourth day. However, on the eighth day, a significant difference was observed between the first and last 2 hours in the control group, but not in the knockout animals (Fig. 7-C). Specifically, male controls consumed an average of 1 g/kg more during the second half of the session compared to the first half. As for female *Bmal1* knockouts, a significant difference was observed between the first and last 2 hours of the fourth day session (Fig. 7-E). Specifically female knockouts consumed an average of 1 g/kg more during the second half of the session compared to the first half. However, this difference was not observed in the controls. On the eighth day of the experiment, no difference was observed among the female mice. This means that male controls and female *Bmal1* knockouts drank more ethanol during the second half of the sessionon the eighth and fourth day respectively.

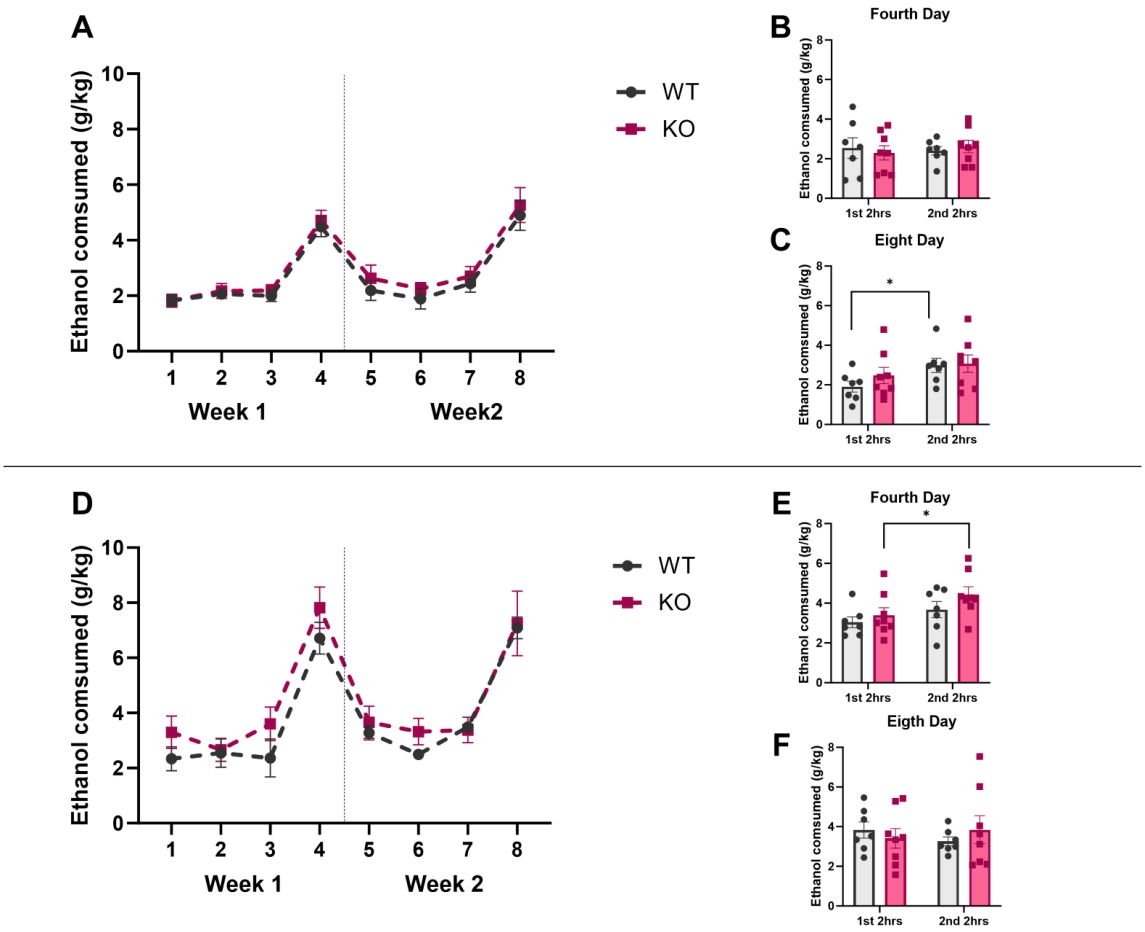

Figure 7. Binge drinking behavior of prefrontal cortex specific *Bmal1* knockouts in the drinking in the dark test. The binge drinking test was used to assess the effect of *Bmal1* knockout in the PFC on alcohol binge drinking in male (A-C, n = 16-19) and female (D-F, n = 7-8) mice. No significant difference in alcohol binge drinking was observed between male *Bmal1* knockouts (A) or female knockouts (D) and their respective controls. On the fourth day of the experiment, male *Bmal1* knockouts (B) and their respective control groups showed no difference in ethanol consumption between the first and second half of the session. However, on the eighth day, a significant difference was observed between the first and last two hours of ethanol consumption in the control male mice (C), but not in the knockouts. In contrast, on the fourth day, female *Bmal1* knockouts (E) had a significant increase in ethanol consumption during the second half of the session compared to the first. Lastly, on the eighth day, female *Bmal1* knockouts (F) and their respective control group expressed no difference in ethanol consumption between the first and the second half of the session. The results are presented as mean ± standard error of the mean. Šidák's multiple comparisons test was performed, with statistically significant differences denoted by * ($p < 0.05$), and ** ($p < 0.005$). Further statistical details can be found in Table 2.

Table 2. *Sucrose preference and binge drinking behavior in male and female mice with Bmal1 knockout in the PFC*

Drinking paradigm		**Two-way repeated measures ANOVA factors**		
Sucrose preference	**Sex**	**Time of Day**	**Virus**	**Interaction**
	Males	$F(1,40)= 5.45$; $p = 0.02$	n.s	n.s
	Females	$F(1,17)= 7.70$; $p = 0.01$	$F(1,17)=5.62$;$p = 0.03$	n.s
Drinking in the dark		**Session**		
Per session	Males	$F(4,95)= 47.32$; $p < 0.01$	n.s	n.s
	Females	$F(3,50)= 49.96$; $p < 0.01$	n.s	n.s

Note. Statistical calculations performed using alpha level of .05. n.s indicates not significant.

Table 3. B*inge drinking behavior on the fourth and eighth day in male and female mice with Bmal1 knockout in the PFC*

		Two-way repeated measures ANOVA factors		
Drinking in the dark	**Sex**	**Per 2 hr interval**	**Virus**	**Interaction**
Fourth day per 2 hrs	Males	n.s	n.s	n.s
	Females	$F(1,13)= 16.19$; $p < 0.01$	n.s	n.s
Eighth day per 2 hrs	Males	$F(1,13)= 12.61$; $p < 0.01$	n.s	n.s
	Females	n.s	n.s	n.s

Note. Statistical calculations performed using alpha level of .05. n.s indicates not significant.

Discussion

This study investigated the impact of *Bmal1* in the PFC on affective-behavior and alcohol binge drinking. The findings indicated that the effect of *Bmal1* deletion in mice is small, however some effects were observed in female mice. Specifically, female *Bmal1* knockout mice showed a decrease in depressive-like behaviors and an increase in anxiety-like behaviors. Conversely, there were no alterations in mood-related behaviors because of the conditional knockout of *Bmal1* in male mice. In relation to alcohol binge drinking behaviors, the absence of *Bmal1* in the PFC did not yield any impact on both male and female mice. Nevertheless, distinctions emerged in the alcohol intake of male and female mice during the initial half compared to the latter half of the four-hour drinking session. To be specific, in the latter half of the four-hour session on day eight, wildtype males consumed more alcohol compared to the first two-hours. Meanwhile, female *Bmal1* knockouts consumed more alcohol during the latter two-hours of the fourth day as opposed to the initial two-hours. Collectively, these findings suggest that disruption in *Bmal1* gene expression in the PFC lead to slight alterations in anxiety- and depressive-like behaviors in female mice, without influencing alcohol binge drinking.

Depressive-like behavior

Depressive-like behavior exhibited variability across experiments. In the sucrose preference test (SPT), the deletion of *Bmal1* in females led to an increased preference for sucrose at ZT 2, indicating an antidepressant response exclusively in females and specifically at ZT 2. Moreover, female control mice displayed a heightened preference for sucrose at ZT 14 compared to ZT 2, a trend that was approaching significance. This time-dependent difference in sucrose preference was not observed in female knockouts. Kant & Bauman (1993) indicated a preference for sucrose that was influenced by the time of day, with the highest preference occurring during the dark phase. This suggests that deletion of *Bmal1* in the PFC might lead to a loss of this innate preference for sucrose during their active phase. In contrast, the absence of *Bmal1* did not impact the behavior of female mice in the tail suspension test (TST) or the novelty suppressed feeding test (NSF). Depressive- like behavior encompasses various facets, and it's possible that the SPT, TST and NSF measure distinct aspects of the same condition (Wang et al., 2017; Planchez et al., 2019). Therefore, a PFC-specific *Bmal1* knockout might affect these behaviors differently.

Additionally, this research observed no discernible variance in depressive- like behavior in male *Bmal1* PFC knockout mice when compared to control subjects. This suggests that the deletion of *Bmal1* in the PFC does not influence depressive-like behavior in males. This contradicts earlier research. Previous studies have indicated an indirect link between *Bmal1* in

the PFC and mood-related behaviors, which was established through the application of induced chronic mild stress (Erburu et al., 2015; Calabrese et al., 2016), simulated night shift (Marti et al., 2017) or neuroinflammation-induced depression (Chen et al., 2020). This was followed by an examination of the impact these induced states of depression have on *Bmal1* rhythms within the PFC. In addition, the animals in the aforementioned studies were subjected to stress, a factor not present in the current experiment, which could explain the divergent findings. However, some studies indicated that deleting *Bmal1* in the forebrain, which encompasses the PFC, did not influence depressive-like behavior (Price et al., 2016).

The effects of *Bmal1* deletion in the PFC may vary depending on sex, given the known sexual dimorphism of these behaviors (Cahill et al., 2021; Lei et al., 2020). Despite the consistent use of behavioral assays, studies often overlook the inclusion of both male and female mice in their sample, potentially missing out on important sex-related distinctions in behavior (Pitzer et al., 2022; Michalidis et al., 2021), like the ones we have identified. This oversight makes it challenging to form a conclusive understanding of how genetic manipulation affects depressive-like behaviors. Furthermore, it is important to mention that the assessment of depressive-like behavior in the TST and NSF was conducted at a single time-point. It has been suggested that timing of testing can influence performance in many behaviors, possibly due to the circadian rhythm of circulating corticosterone levels (Sare et al., 2021). Considering this, further studies should consider conducting the TST and NSF at different time points to ascertain whether behavioral disparities are contingent on the time of day.

The PFC and the striatum are closely connected both structurally and through direct glutamatergic projections, which trigger local dopamine release in the striatum (Alexander et al., 1986; Haber et al., 2006; Adrover et al., 2020). Abnormalities in dopamine signaling are implicated in various psychopathologies, including affective disorders and addiction (Chung et al., 2014; Koob & Volkow, 2010). While dopaminergic signaling in the PFC is involved in cognitive control, working memory and emotional response (D'Ardenne et al., 2012; Weele et al., 2019; Arnsten et al., 2015), those within the striatum are associated with motor impairments such as those seen in Parkinson's disease, which can trigger depression (Brichta & Greengard. 2014). Previous research has indicated that disruptions in the functional connectivity between these areas are associated with certain mental disorders like depression and alcohol dependence (Zhang et al., 2016; Heller el al., 2009; Coutney et al., 2013). Given this and considering that both brain regions exhibit rhythmic expression of clock genes (Christiansen et al., 2016; Cai et al., 2009), it is imperative to further study the impact of *Bmal1* deletion in both

regions and their interconnectedness, as well as the interaction of clock genes within the PFC on dopamine signaling and behavior changes.

The precise mechanism through which *Bmal1* influences depressive-like behavior in the PFC remains unclear. It is important to consider other clock genes, as demonstrated by Otsuka et al. (2022), who found that mice lacking Rev-erbα exhibited reduced serotonin levels in the PFC. Serotonin is strongly implicated in mood regulation. Nevertheless, the findings of this study lend support to the idea that clock genes within the PFC play a role in depressive disorders.

Anxiety-like behavior

The conditional knockout of *Bmal1* in the PFC led to a heightened anxiety-like behavior in females. More precisely, females with *Bmal1* deletion had an increased latency to enter the center of the open field, indicating an increase in anxiety-like behavior. Conversely, males with *Bmal1* deletion in the PFC did not exhibit any discernible difference in anxiety-like behaviors compared to the control group. Ostuka et al. (2020) demonstrated that inducing an anxious state through shift work models altered *Bmal1* expression in the PFC. In contrast, Price et al. (2016) found that *Bmal1* knockout in the forebrain, which includes the PFC, did not influence anxiety-like behavior. Additionally, Kondratova et al. (2010) did not observe any disparities in anxiety-like behavior between *Bmal1* knockout and control groups. These distinctions may be attributed to the specific removal of *Bmal1* within the PFC. Moreover, sex differences observed in anxiety-like behavior can be linked to sex differences in the robustness of clock gene expression. Chun et al. (2015) indicated that females exhibit less robust rhythms in the medial PFC compared to males, suggesting that gonadal hormones might regulate the expression of molecular clock genes, potentially accounting for the disparities between the sexes.

Ethanol binge-drinking behavior

The findings indicated that specific deletion of *Bmal1* in the PFC has no discernible impact on ethanol binge-drinking behavior in male and female mice when compared to control groups. This suggests that the mechanism involving *Bmal1* in the PFC does not play a significant role in binge drinking in both sexes. Additionally, in the control group, male mice displayed a significant difference in ethanol intake between the initial and final two hours on the eighth day, which was not observed in the *Bmal1* knockout group. Specifically, there was a higher ethanol consumption during the latter half of the four-hour period. However, this pattern was not evident on the fourth day. Regarding female *Bmal1* knockout mice, a significant disparity was observed between the initial and final two hours of the fourth day session. Female knockouts consumed more ethanol during the second portion of the session compared to the

first half. This pattern was not seen on the eighth day. Previous research has established that both experimental and control mice tend to consume more ethanol during the latter half of the four-hour session as opposed to the first half (Barkley-Levenson & Crabbe., 2012). However, since this pattern was not consistent across all days and groups in our study, further research is warranted to draw more definitive conclusions.

In general, there exists a bidirectional relationship between ethanol abuse and circadian rhythm disruption. However, the precise mechanism through which clock genes influence both ethanol binge drinking, and abuse remains unclear. Previous research indicated an effect of ethanol on the expression of clock genes in the PFC. For example, Del Olmo et al. (2019) observed altered expressions of *Clock*, *Per2* and *Bmal1* within the PFC in mice following ethanol binge drinking and high-fat diet consumption. Additionally, Lindberg et al. (2018) found that ethanol consumption entrains *Per 1* expression rhythms in the PFC. Previous research has demonstrated that ethanol impacts neural functions by influencing various neurotransmitter systems, including GABA, glutamate, norepinephrine, and dopamine (Weiner & Vanlenzuela. 2006, Weinshenker et al. 2000, Woodward 2000, Parekh et al. 2015). The primary neurotransmitter implicated appears to be glutamate, primarily released from projections originating in the frontal regions and basolateral amygdala to the nucleus accumbens (Koob & Volkow. 2016, Tamura et al. 2021). Hence, future studies should explore the influence of *Bmal1* on glutamatergic signaling within the PFC, as well as its interplay with reward systems like the nucleus accumbens, in the context of ethanol binge drinking. Additionally, the transition to addiction is believed to entail neuroplasticity across all limbic regions and is thought to progress through a series of dysfunctions, commencing with dopamine signaling in the VTA and affecting target regions including the striatum and PFC (Koob & Volkow. 2010). This facilitates the shift from use to abuse and eventually dependence. Consequently, future studies should delve into the consequences of *Bmal1* knockout within the PFC on other aspects of ethanol consumption, such as preference. This holistic approach will provide a more comprehensive understanding of the effects of *Bmal1* on ethanol-related disorders.

Limitations

It’s worth noting that conflicting findings might be attributed to the ongoing debate surrounding animal behavioral testing. Carter et al. (2013) highlighted a criticism of testing, noting that there are numerous tests designed for a single trait. This arises from the fact that these tests are not always directly comparable, lacking standardization in assessing the same behavior. Additionally, tests may measure entirely different constructs. Another issue arises when one test assesses multiple traits. It becomes challenging to discern which trait is truly

being assessed and what kind of interactions may be at play. Hence, it is advisable to incorporate other behavioral tests such as the elevated plus maze to gain a broader perspective on the impact of *Bmal1* on anxiety-like behavior. Furthermore, it's crucial to consider individual variation among subjects, as well as any correlations between individuals across different tests, to determine if the observed phenotype is consistently reproducible.

As previously mentioned, to gain a more comprehensive understanding of ethanol consumption, it is imperative to assess more than just binge drinking behavior. Conducting a two-bottle choice test would be crucial to investigating ethanol preference, providing a more complete perspective on how clock genes in the PFC impact this behavior. Osterndorff-Kahanek et al. (2013) indicated that the drinking in the Dark model is the least representative of chronic alcohol consumption, but it remains a relevant model of binge drinking, involving the activation of brain structures other than PFC. In contrast, the chronic intermittent paradigm (two-bottle choice) serves as a robust model for studying alcohol consumption in the PFC. Additionally, it would be valuable to investigate the response to other substances of abuse, given that prior research has indicated that *Bmal1* knockout mice demonstrated reduced seeking behavior for substances like cocaine (Castro-Zavala et al., 2022). The PFC is densely populated with both Dopamine 1 (D1) and Dopamine 2 (D2) receptors. Research has demonstrated that these receptors have distinct roles in influencing behavior (Arnsten et al. 2015, Jenni et al. 2017). For instance, St Onge et al. (2011) proposed that blocking D1 receptors in the PFC diminishes risky/reward-based decision-making, while the D2 antagonist heightens it. Furthermore, evidence suggests that there are sex-specific differences in dopamine D1-D2 receptor complex, influencing behaviors related to depression, anxiety behaviors (Hasbi et al., 2020), and addiction (Williams et al., 2021). This indicates that differences in mood and ethanol binge drinking may not only be explained by region-specific factors, but also by cell type-specific influences. Therefore, forthcoming studies should investigate D1 and D2 cell type-specific knockouts within the PFC, while considering the influence of sex, behavioral tests, and efficiency of the virus.

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