Nucleus accumbens specific knockout of *Bmal1* and *Per2* regulates mood-like behaviors and alcohol consumption in male and female mice

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ABSTRACT

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Maria Juliana Herrera Barrera

Disrupted clock gene expression, either in the central clockwork or in peripheral brain areas, is known to affect behavior. Given the comorbidity between affective disorders and alcohol use disorder in males and females, understanding the role of clock genes is necessary to improve drug treatments. The function of clock genes within striatal medium spiny neurons has gained attention for its region-specific associations with neuropsychiatric disorders. Until now, how core clock genes within specific subdivisions of the striatum affect mood and addiction disorders in male and female mice is not fully understood. We measured anxiety-like and depressive-like behaviors as well as ethanol consumption in male and female mice with nucleus accumbens deletion of *Bmal1* or *Per2*. Whereas deletion of *Bmal1* in the ventral striatum had minor effects on mood-related behavior, it had significant positive effects on ethanol drinking in both males and females, suggesting that *Bmal1* in the nucleus accumbens normally suppresses alcohol consumption independent of sex. Deletion of *Per2* augmented alcohol consumption in males, mimicking the effect of *Bmal1* nucleus accumbens knockout. The same deletion had no effect in females, pointing to female-specific dissociation between the effect of nucleus accumbens *Bmal1* and *Per2* on alcohol intake. This study provides new insight into the role of clock genes in the nucleus accumbens in the control of alcohol drinking behavior in male and female mice. Furthermore, lack of effect of *Bmal1* and *Per2* deletion on mood related behaviors in these mice suggests that the effect on alcohol consumption is direct and not due to changes in mood state.

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CONTRIBUTION OF AUTHORS

Experiments were designed by Dr. Shimon Amir, Dr. Konrad Schöttner, and Juliana Herrera. Surgeries and behavioral experiments were conducted by Juliana Herrera and assisted by Margo Button. Ethanol experiments were assisted by Pascale Doherty-Haigh. Data was analyzed by Juliana Herrera. Data visualization was done by Juliana Herrera. Manuscript production was written by Juliana Herrera, with editing from Dr. Shimon Amir, Dr. Konrad Schottner, and Margo Button.

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One in four individuals over the age of 18 is affected by a psychiatric or substance abuse disorder at some point in their lifetime (Steel et al., 2014; Ross & Peselow, 2012; Kelly & Daley, 2013; Kessler et al, 2005). Affective disorders such as depression and anxiety are strongly comorbid with alcohol-use disorders and pose a threat to an individual's quality of life. In recent years, circadian clock genes emerged as potential regulators of several aspects of mood abnormalities (Albrecht, 2020) and alcohol consumption (Partonen, 2015). Gene association studies in humans link core clock genes to alcohol consumption and heightened risk of alcohol use disorders (Kovanen et al., 2010; Valenzuela et al., 2016). Similarly, circadian gene variants are associated with bipolar disorder, major depressive disorder, and seasonal affective disorder (Kripket et al., 2009; Mansour et al., 2006; Soria et al., 2010). Understanding the role of clock genes in mood and alcohol use disorders is necessary to improve diagnosis and treatments.

The master circadian clock is located in the suprachiasmatic nucleus (SCN). Its primary function is to generate 24-hour rhythms and synchronize physiological and behavioral processes to the external environment. A family of core clock genes form interlocking feedback loops that generate these intrinsic rhythms by driving daily cycles of expression, accumulation, and auto degradation of clock gene products. At the cellular level, the primary transcription-translation feedback loop (TTFL) consists of Brain and Muscle Arnt-Like 1 (*Bmal1*) and Circadian Locomotor receptor Output Cycles Kaput (Clock), who's protein products heterodimerize to initiate the transcription of *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*) genes via E-box enhancers in their promoters (Takahashi et al., 2017). In turn, PER and CRY proteins inhibit the transcriptional activity of BMAL1/CLOCK and ultimately repress their own transcription. Degradation of PER and CRY ceases their repressing function so that the cycle of transcription/translation can start again. The secondary loop maintains the transcription of *Bmal1* through *Retinoid orphan receptor* (*ROR-α, β, γ*) and *Rev-Erb-α/β*. Clock genes are an integral part of the molecular clockwork and therefore any disruption to their expression can affect cellular functions and result in pathological conditions, including psychiatric disorders (Rosenwasser 2010).

Under the control of the central pacemaker in the SCN, other brain areas rhythmically express clock genes (Cai et al., 2009; Salaberry et al., 2019). Two such areas are the dopaminergic mesolimbic and nigrostriatal regions. The mesolimbic region consists of the ventral tegmental area (VTA) and the nucleus accumbens (NAc), a ventral striatal region implicated in motivation, mood, and reward (Nestler and Carlezon, 2006). The nigrostriatal region consists of the substantia nigra (SN) and dorsal striatum, a region best known for its role in the control of motor functions and some aspects of reward (Wise, 1998). Many psychopathologies including addiction and affective disorders are linked to abnormalities in striatal dopamine signaling. While the mesolimbic pathway is related to mood and addiction (Grace, 2016; Hyman and Malenka, 2001), motor deficiencies like Parkinson's disease, which can lead to depression, are associated with a degenerated nigrostriatal pathway (Cheng et al., 2010). Importantly, these striatal regions have rhythmic expression of clock genes (Guilding and Piggins, 2007; Chung et al., 2014) that control vital neurotransmitter systems (Castaneda et al., 2004). For instance, the release of dopamine, glutamate, and gamma-aminobutyric acid (GABA) within the NAc, a brain region firmly associated with mood and addiction disorders, is under clock gene control (Castaneda et al., 2004; Weber et al., 2004; Webb et al., 2009). Inversely, clock gene expression within the striatum is sensitive to dopamine release (Hood et al., 2010). This evidence suggests circadian clock gene expression and dopamine signaling mutually influence each other to potentially regulate affective disorders and aspects of substance abuse.

Human studies support the relation between clock genes and mesolimbic related mood and reward disturbances. For instance, in postmortem brains of individuals diagnosed with major depressive disorder (MDD) circadian gene expression was disrupted in the NAc (Li et al. 2013). Similarly, a downregulation of clock-controlled genes within the NAc was observed in brains of suicide victims (Sequeria et al., 2012; Li et al., 2017). Common pharmaceutical treatments for MDD target monoamine neuromodulators (Xu et al., 2020), which are regulated by clock proteins *BMAL1* and *PER2* (Hampp et al., 2008). Polymorphisms in the human *Clock*, *Bmal1*, and *Nr1d1* genes are associated with mood-related phenotypes (Etain et al., 2011) while polymorphisms to the human *Per2* gene are associated with higher alcohol intake (Spanagel et al., 2005). Thus, the accumulated evidence suggests that functional expression of circadian clock genes in humans is compromised in depression and alcohol drinking disturbances.

Studies in rodents emphasize the effects of mesolimbic and nigrostriatal clock gene knockout on anxiety-like and depressive-like states as well as alcohol intake. Schöttner et al. (2022) reported moderate anxiety-like and depressive-like behaviors in mice with a conditional knockout of *Bmal1* in striatal medium spiny neurons (MSNs). Mice with NAc specific knockdown of *Per1* and *Per2* genes display higher anxiety-like behavior (Spencer et al., 2013). Similarly, chronic social defeat stress increased anxiety-like behavior and decreased *Per1* and *Per2* gene expression in the NAc (Spencer et al., 2013). In relation to alcohol consumption, Spanagel et al. (2005) reported that global *Per2* mutations in mice increases alcohol consumption and preference. Chen et al. (2011) documented how chronic alcohol exposure alters clock-controlled neurotransmission onto MSNs. de Zavalia et al. (2021) demonstrated sex-specific alterations in alcohol consumption in conditional knockout mice lacking *Bmal1* exclusively in striatal-MSN. Therefore, an interplay exists between clock genes in the striatum, mood, and alcohol drinking behaviors.

Growing evidence suggests the comorbidity of circadian clock gene disturbances in depression, anxiety, and alcohol consumption. Recent studies found striatal specific alterations of clock genes to be the cause for these conditions, perhaps by the interaction with the dopamine signaling pathway (Kim et al., 2017). Since the NAc and dorsal subregions of the striatum exhibit functional and neuroanatomical differences, this study aimed to narrow down the behavioral effects of *Bmal1* and *Per2* knockout to the NAc. We hypothesized that alterations to clock gene expression within the NAc would alter behaviors associated with anxiety, depression, and alcohol drinking.

Methods

Animals

This study utilized 12 to 16-week-old male and female transgenic mice bearing floxed alleles of either the *Bmal1* (*Bmal1*$^{fl/fl}$, Jackson Laboratory, stock number 007668) or *Per2* (*Per2*$^{fl/fl}$, European Mouse Mutant Archive, Strain ID: EM10599) gene. Mice were group-housed (2-4 subjects) until the day of stereotactic surgery, under a strict 12-h light/12-h dark cycle regime with controlled room temperature 21 ± 1°C and access to food and water *ad lib*. After stereotactic surgery, mice were individually housed in light- and soundproof boxes under similar conditions as described above. All animal procedures conformed to the guidelines of the

Canadian Council on Animal Care and by the Animal Care Committee of Concordia University (certificate number: 30000256).

Intracerebral Injections of Adeno Associate Virus

Bmal1 and *Per2* floxed mice underwent bilateral stereotactic injections to the nucleus accumbens (NAc). 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 (AAV2/5-CAG-EGFP, 1.0 x 10^{12} vg/ml, Molecular Tool Platform, Quebec, Canada) were delivered to the NAc, hereafter referred to as NAcKO and NAcCNTRL, respectively. Surgery coordinates targeting the NAc were anteroposterior: 1.6 mm posterior to bregma, mediolateral: 1.00 mm lateral to midline, dorsoventral: -4.7 mm ventral to the skull (taken as "0") (Paxinos & Franklin, 2001). Virus was injected at a rate of 250 nl/min. Behavioral procedures were conducted 3 weeks after viral vector delivery (Figure 1A).

Behavioral procedures

To test for anxiety- and depressive-like behaviors the Elevated Plus Maze (EPM), Open Field Test (OFT), Marble Burying Test (MBT) and the Tail Suspension Test (TST) were conducted with at least one week interval between tests. To assess voluntary ethanol consumption and binge drinking behavior the intermittent access to ethanol (IAE) and drinking in the dark (DID) tests were used. All behavioral procedures were run between ZT 2-6 (i.e. 2 – 6 h after lights-on), except for the tail suspension test conducted at ZT8 and the drinking in the dark at ZT15. Mice were habituated to the testing environment one hour before starting the experiment. Body weights were recorded after each behavioral test. Vaginal smears were collected after the completion of behavioral experiments and on the first and last day of the drinking in the dark test to determine female estrous stage.

Measures

Elevated Plus Maze

The elevated plus maze was used as an assay for anxiety-like behaviors. The "+"-shaped maze sits 40cm above the ground with two enclosed arms (6 x 29.5 cm), two open arms (6 x 29.5cm) and a center area (6 x 6 cm). White methacrylate floors and black walls (15 cm in height) lined the bottom and enclosed arms of the maze, respectively. The test is based on mice's natural aversion to heights and open areas (Walf and Frye, 2007). Mice were habituated to the testing room by leaving them in their cage for an hour before the start of the experiment. Then,

the mouse was placed in the center area of the elevated plus maze (Harvard Apparatus, Holliston, MA, USA) facing the closed arm and allowed to explore the maze for 5 minutes while video recorded (Samsung Galaxy A5 2017 phone). Video files were analyzed as described in Schoettner et al. (2022).

Open Field Test

The aim of the open field test was to measure anxiety-like behaviors by capitalizing on mice's innate aversion to large, open, and unknown environments (Seibenhener and Wooten, 2015). After an hour of testing room habituation inside the home cage, subject activity was recorded for 60 minutes by the Actitrack software package (Panlab, Barcelona, Spain) in an open field arena equipped with infrared beams (width 45cm, depth 45cm, height 50cm) and surrounded by plexiglass walls (44 x 44 x 30cm). The total distance traveled, time spent in the center of the arena, and latency to enter the center of the arena were assessed.

Marble Burying Test

The marble burying test was used to measure anxiety-like behavior (Deacon 2006). After an hour of home cage habituation inside the testing environment, mice were placed in the corner of a large housing cage (43 x 21 x 21 cm) filled with 5 cm of Sani-Chip bedding. Twenty different colored glass marbles (Ø16mm, Editions Gladius International, Quebec, QC, Canada) were lined up in 4 vertical rows 7.5cm away from each other before introducing the mouse. The mouse was left inside the cage for thirty minutes. When the test was complete, the number of buried and unburied marbles was recorded. A marble was considered buried if two-thirds of its surface area was covered in Sani-Chip.

Tail Suspension Test

The tail suspension test was used to assess depressive-like behavior (Can et al., 2012). After an hour of testing room habituation within the home cage, the mouse was suspended by the tip of the tail to a metal bar that sits 30 cm above the ground with adhesive tape. Plastic tubing was placed above the tail to prevent the animal from climbing or injury. Mice were suspended for a total of 6 minutes while video was recorded (Samsung Galaxy A5 2017 phone). Mobility time was analyzed using the Stopwatch+ software (Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA, USA). Immobility time was calculated by subtracting mobility time from total experiment time.

Intermittent Access to Ethanol (IAE)

An intermittent two-bottle choice paradigm was used to measure ethanol consumption and preference (Hwa et al., 2011). Individually housed mice were habituated to the experimental setup with two water bottles with ball bearing rubber stoppers three days before the start of the 11 sessions. Every other day, mice were given one bottle of water and one bottle of 15% (v/v) ethanol in tap water for 24 hours for 11 total ethanol access sessions. The position of the ethanol bottle alternated (right or left) each session to avoid any side preference confound. As a control, empty cages with the same bottle set up were used to measure any potential leakage or evaporation of liquid. Ethanol consumption by bodyweight (g/kg) was analyzed by multiplying the weight of alcohol consumed (g) by the density of the alcohol concentration then divided by the weight of the mouse in kilograms. Alcohol preference was calculated as the amount of alcohol intake divided by the total fluid intake (alcohol intake (g)/ alcohol intake (g) + water intake (g)).

Sucrose Preference Test

Immediately following the IAE, mice were again habituated to two bottles of tap water with ball bearing rubber stoppers for three days. Then, mice were exposed to one bottle of tap water and one bottle of 0.25% (m/v) sucrose in tap water for 24-hours with three consecutive sessions. The bottles were weighed before and after each session. The ratio of the sucrose solution intake relative to the total intake of liquid was analyzed as a measure of taste preference.

Drinking in the dark test

To assess binge drinking behavior a 4-day drinking in the dark paradigm was conducted (Thiele et al., 2014) with one group of mice naïve to ethanol and one group preexposed to IAE four to twelve weeks earlier. In brief, both experienced and naïve mice were given a bottle of 20% ethanol three hours into the dark cycle (ZT15) for 2 hours on the first three days, and four hours on the fourth day. Trunk blood was collected immediately after the fourth day drinking session for blood ethanol concentration analysis. Brains were flash-frozen after dissection and stored in -80 °C. Body weights and vaginal lavage (McLean et al., 2012) were collected during lights-on on the first and last day of the experiment.

Viral Infection Validation

Brains were fixed by transcardial perfusion with cold saline (0.9% NaCl) and paraformaldehyde (4% in 0.1M phosphate buffer, pH 7.3). Following a 24-hours post-fixation period at 4°C, 30µm coronal slices of brain tissue were collected using a Leica vibratome and

analyzed under a fluorescent microscope to validate region-specific viral vector delivery. Mouse subjects without GFP signaling within the NAc core or shell were eliminated from the analyses. Figure 1B and 1F depicts the overlap of GFP expression for all knockout and control surgeries. Brain slices were stored at -20°C in Watson's cryoprotectant (Watson et al., 1986) for immunofluorescence imaging.

Immunofluorescence

Free-floating sections previously kept in Watson's cryoprotectant were rinsed for 10 minutes in phosphate buffered saline (PBS, pH 7.4) and then rinsed three time in 0.3% Triton-X in PBS (PBST) for 10 minutes each. Immunofluorescent staining with an Anti-Cre (1:2000, C7988, Sigma-Aldrich) and Bmal1 (1:500, NB100-2288, NovusBiologicals) antibodies were used to determine viral infection efficiency of *Per2* and *Bmal1* knockouts, respectively. A ratio between CRE/GFP expressing cells over total number of DAPI stained cells was used to calculate rate of viral infection. Representative images were prepared using FIJI (Schindlein et al., 2012) and Inkscape (Inkscape Project, 2020).

Statistical Analysis

Statistical analysis was conducted using Prism 9 (GraphPad Software, San Diego, CA, USA). Values are reported as means ± standard error of mean (SEM). Behavioral tests were analyzed using unpaired two-tailed t-tests for between group comparisons (NAcCNTRL vs NAcKO). Ethanol related procedures were analyzed by two-way repeated measures ANOVA (virus by session) followed by post-hoc analysis of significant main effects. In all analyses, the null hypothesis of no model effects was rejected at $p < .05$.

Results

Validation of nucleus accumbens specific clock gene knockout

Nucleus accumbens (NAc) knockout efficiency and specificity were validated by immunofluorescent staining (Figure 1-3). Image analysis of DAPI (Figure 2, 3A), Green Fluorescent Protein (GFP; Figure 2, 3B) and Cre recombinase (Cre; Figure 2, 3C) revealed that cells in the NAc were infected with virus expressing Cre and GFP, in the case of clock gene knockouts (NAcKO), or GFP only in the case of controls (NAcCNTRL) (Figure 2,3E-G). Cell counting procedures on merged images (Figure 2, 3E) revealed that on average 65-75% of cells within the NAc were infected with Cre or empty virus

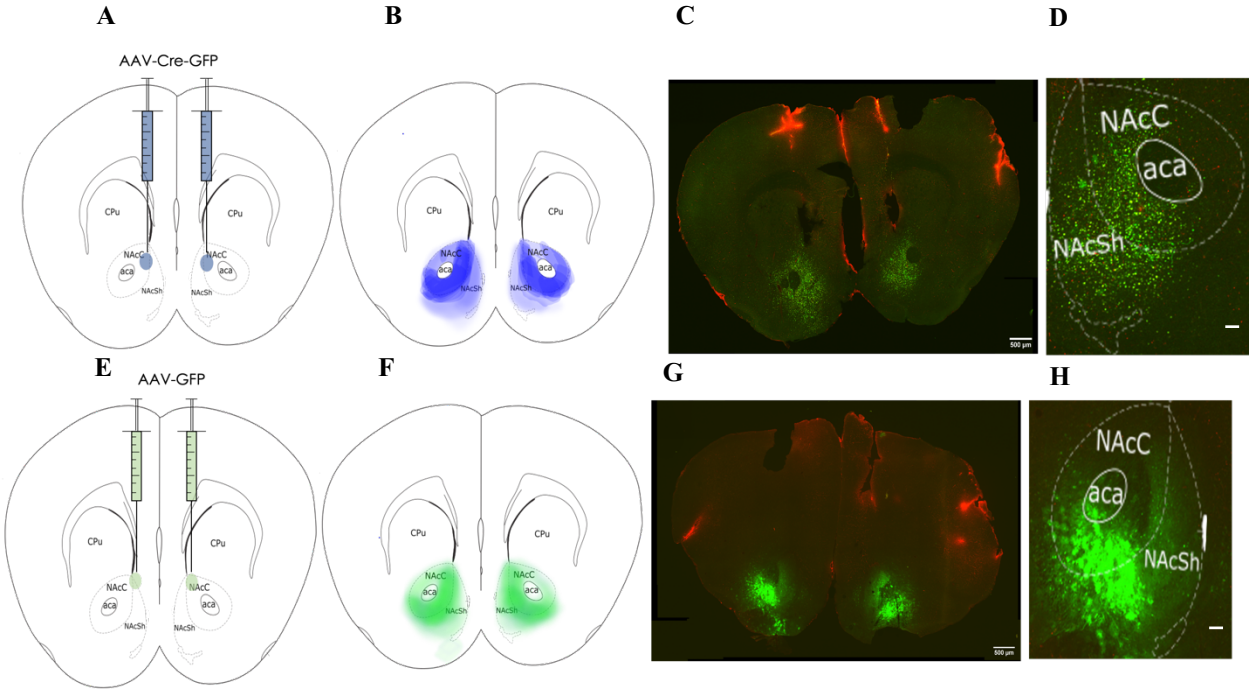

Figure 1. Representative images of viral knockout validation.
Schematic depicting the injections of AAV-Cre-GFP (A) or AAV-GFP (E) to the NAc. Schematic drawings depicting the overlap of validated NAcKO (B) and NAcCNTRL (F) surgeries. Images produced by immunofluorescent staining show coronal sections with exclusive expression of cre (C) and control (G) virus within the NAc. Representative image depicting GFP expression within the subdivisions of the NAc of knockout (D) and control (H) mice, scale bar 50 µm.

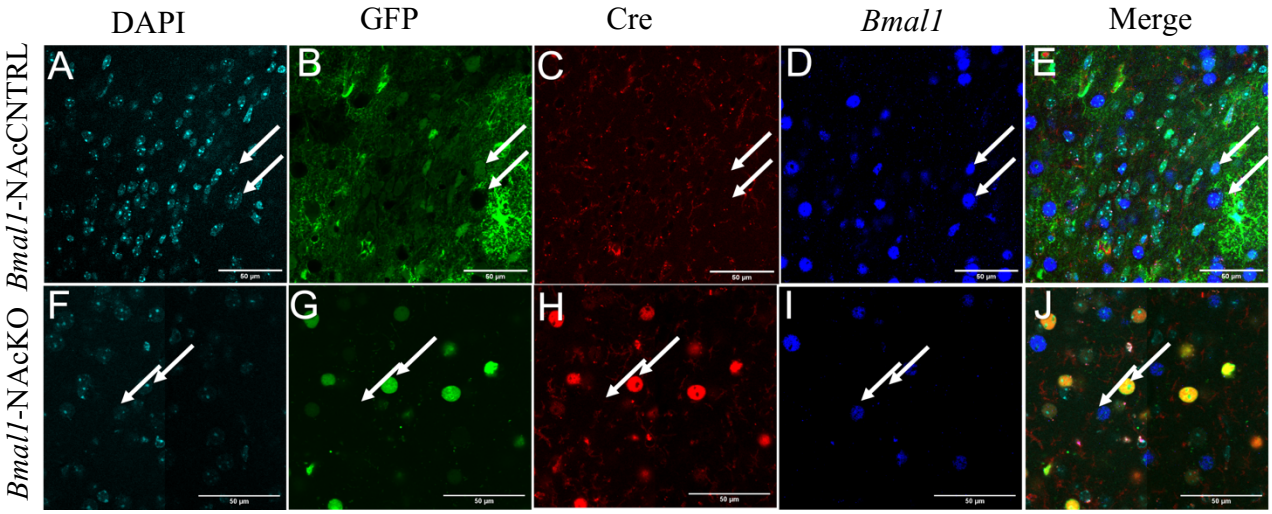

Figure 2. *Bmal1* nucleus accumbens viral knockout and control validation
Representative images of labeled cells in the nucleus accumbens of *Bmal1* controls (A-E) and knockouts (E-H). Arrows point to one cell infected with virus and one without. DAPI= blue, GFP= green, Cre-Recombinase = Red.

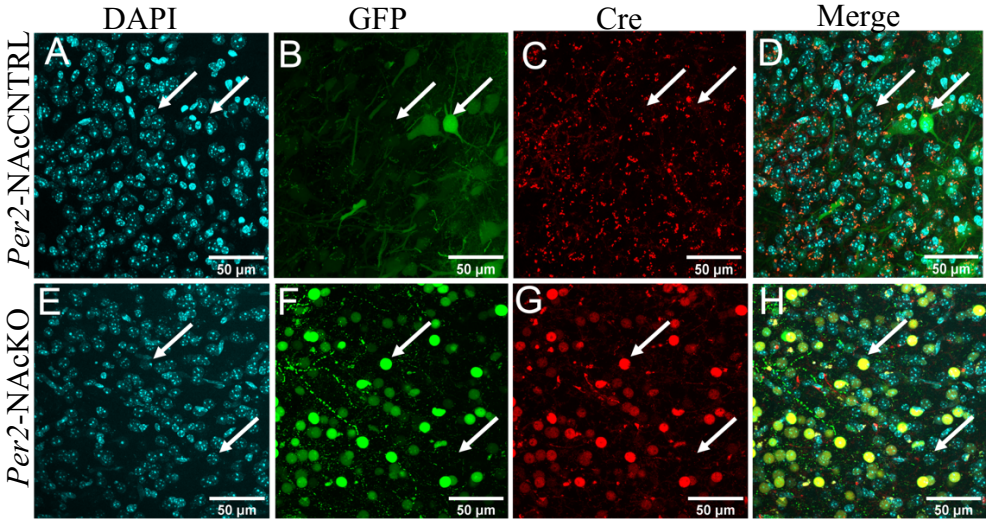

Figure 3. *Per2* nucleus accumbens viral knockout and control validation
Representative images of labeled cells in the nucleus accumbens of *Per2* controls (A-D) and knockouts (E-H). Arrows point to the same cells per panel. DAPI= blue, GFP= green, Cre-Recombinase= Red.

Anxiety-like behavior of *Bmal1* and *Per2* nucleus accumbens knockout

To test if the absence of *Bmal1* or *Per2* within the NAc affects anxiety-like behaviors in male and female mice, clock gene knockouts and controls were assessed using the Elevated Plus Maze (EPM) (Figure 4), Open Field Test (OFT) (Figure 5), and Marble Burying Test (MBT) (Figure 6).

Bmal1 NAc knockout (-NAcKO) did not affect anxiety-related behaviors in either male or female mice regardless of behavioral experiment (Figure 4-6). Similarly, no changes in anxiety levels were found in males with NAc-specific deletion of *Per2* (Figure 4-6). Detailed statistics are summarized in Table 1.

Female mice with *Per2* ablation in the NAc displayed less anxiety-like behavior in the EPM (Figure 4). Specifically, female *Per2*-NAcKO spent 32% more time in the open arm of the EPM and frequented the open arm by 11% more than controls. Total distance traveled in the EPM was unaffected by the clock gene manipulation. Whereas an anxiolytic effect of the knockout was observed in the EPM, an anxiogenic tendency was observed in the OFT (Figure 5). On average, *Per2*-NAcKO females took 48 seconds longer to enter the center of the open field compared to controls, however, knockout and control groups did not differ in the total amount of time spent in the center of the open field or total distanced traveled. Lastly, anxiety like behavior measured in the MBT (Figure 6) was unaffected by the female *Per2* NAc knockout.

Depressive-like behavior of *Bmal1* and *Per2* nucleus accumbens knockout

To assess the role of NAc clock gene knockout on depressive-like behaviors, the tail suspension test (TST) was conducted (Figure 7). No differences in immobility time were observed in female *Bmal1*-NAcKO or male *Per2*-NAcKO compared to their respective controls. Detailed statistics are listed on Table 1. While *Bmal1*-NAcKO affected depressive-like behaviors in males, *Per2*-NAcKO affected depressive-like behavior in females. An unpaired two tailed t-test revealed that on average, *Bmal1*-NAcKO males spent 13.25% less time immobile than controls when suspended by the tail on a metal bar. In contrast, female *Per2*-NAcKO spent 26% more time immobile than controls on the TST.

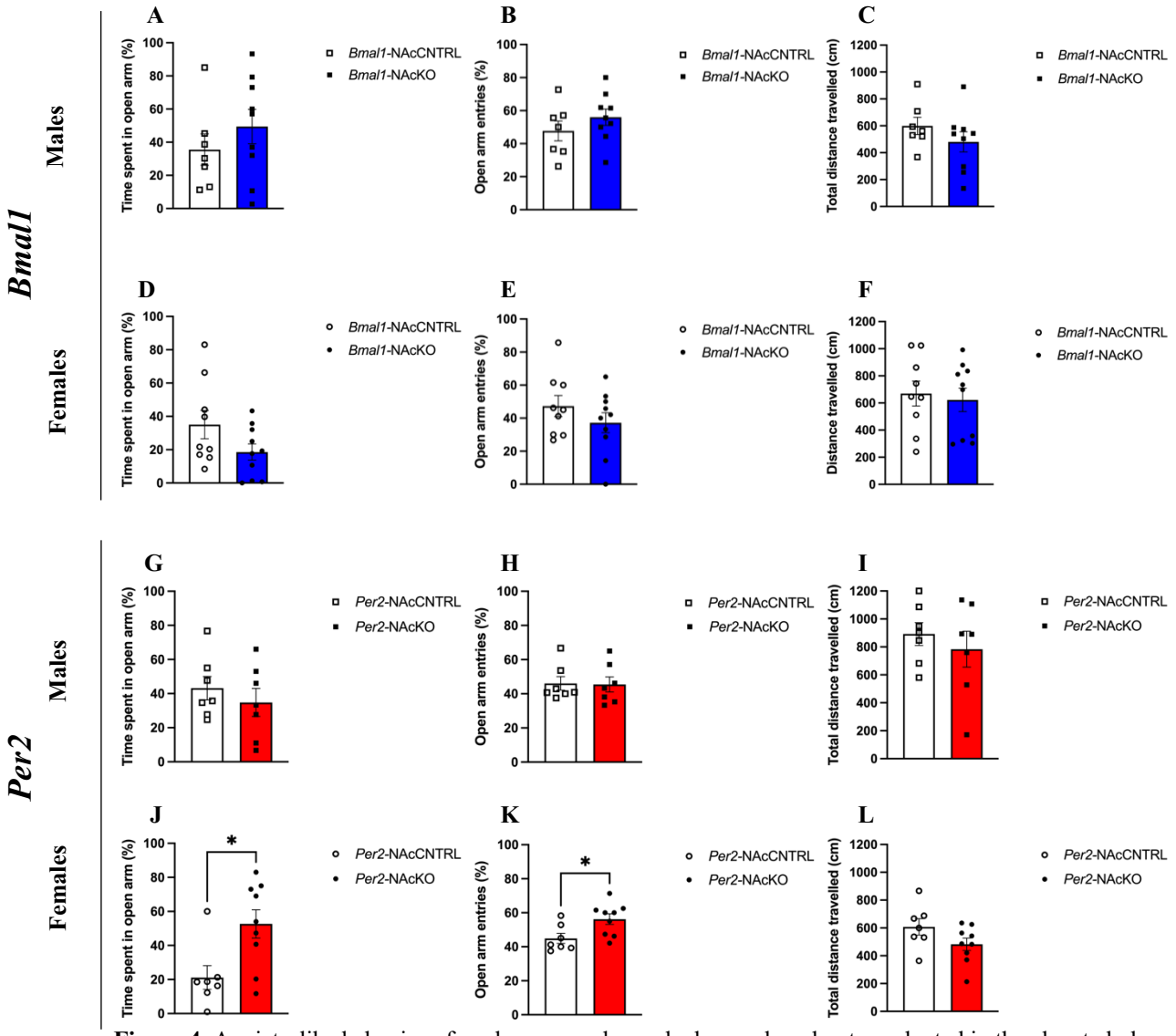

Figure 4. Anxiety-like behavior of nucleus accumbens clock gene knockouts evaluated in the elevated plus maze. In the elevated plus maze time spent in the open arm, open arm entries, and total distance traveled was measured in male (squares) and female (circles) nucleus accumbens clock gene knockouts. No difference in anxiety-like behavior was observed in male (A-C n = 7-9) or female (D-F n = 8-9) *Bmal1*-NAcKO and controls. Whereas no changes in anxiety-like behavior was displayed between male (G-I n= 7) *Per2*-NAcKO and controls, female (J-K n = 7-9) *Per2*-NAcKO entered and spent more time in the open arm of the EPM compared to controls. Total activity levels were unaffected (L). Results are depicted as mean ± standard error of the mean. *…$p < 0.05$. Details of independent measures t-test summarized in Table 1.

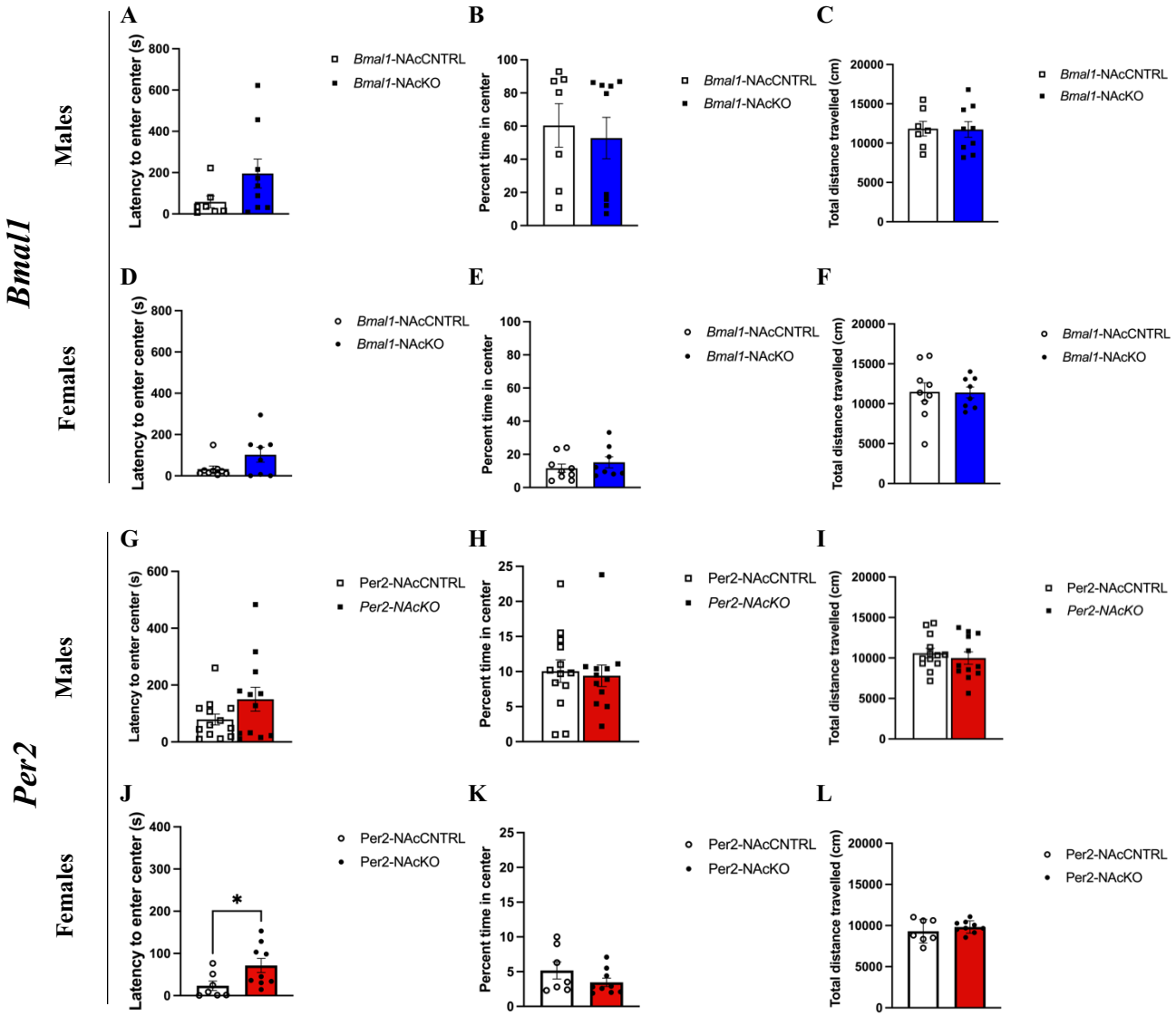

Figure 5. Anxiety-like behavior of nucleus accumbens clock gene knockouts assessed by the open field test. In the open field test latency to enter center, time spent in the center, and total distance traveled was measured in male (squares) and female (circles) nucleus accumbens clock gene knockouts. No difference in anxiety-like behavior was observed in male (A-C n = 7-9) or female (D-F n = 8-9) *Bmal1*-NAcKO and controls. Although no changes in anxious behavior was observed in male (G-I n = 12-13) *Per2*-NAcKO, there was an increase in the latency to enter the center of the open field in female (J n = 7-9) *Per2*-NAcKO compared to controls. Percent time in the center and total distance traveled remained unaffected (K-L). Results are depicted as mean ± standard error of the mean. *…p < 0.05. Details of independent measures t-test summarized in Table 1.

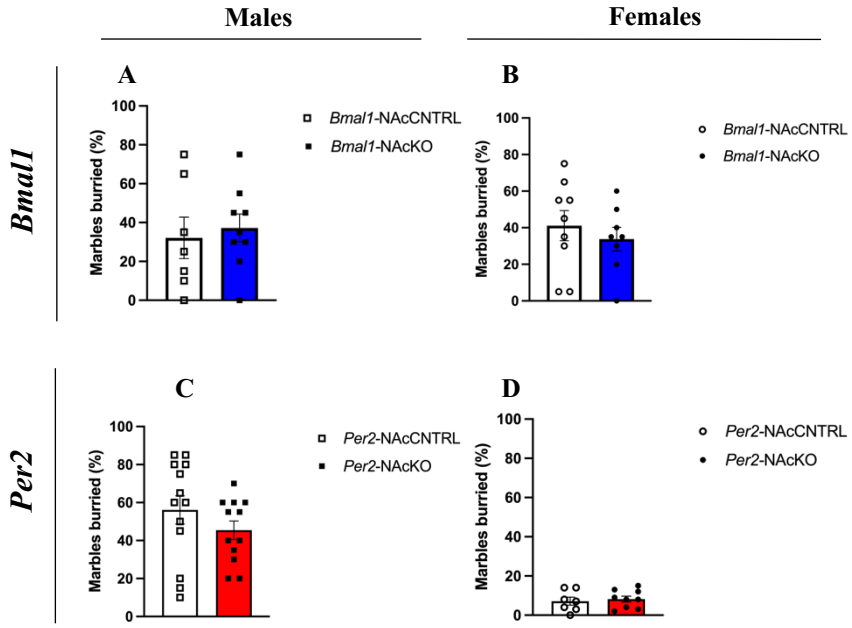

Figure 6. Anxiety-like behavior as measured by the marble burying test in nucleus accumbens clock gene knockout. Number of marbles buried out of 20 total marbles was measured in male (squares) and female (circles) clock gene knockouts. No differences in percent marbles buried was observed in either clock gene knockout (A-D). Results are depicted as mean ± standard error of the mean. B n = 8-9. C n = 12-13. D n = 7-9. Detailed statistics summarized in Table 1.

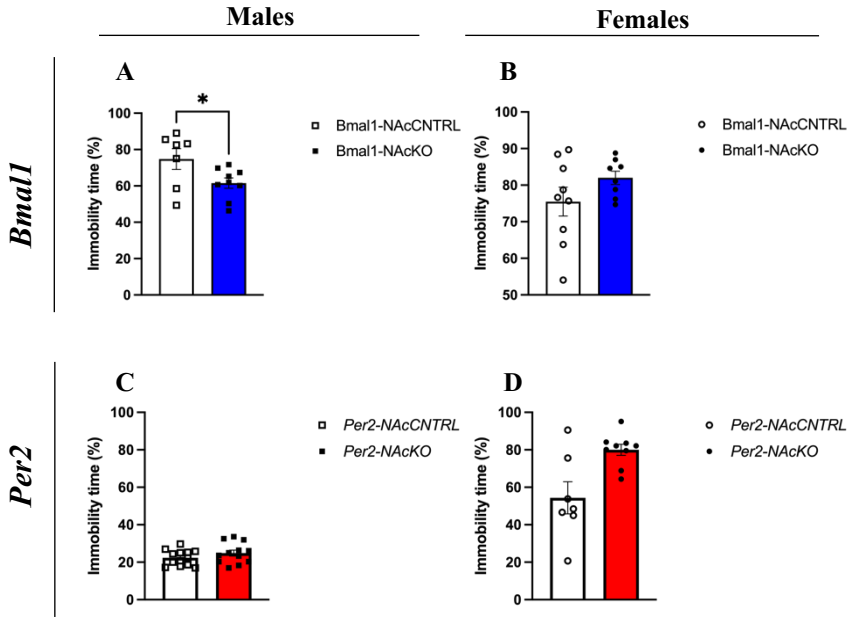

Figure 7. Depressive-like behavior of nucleus accumbens clock gene knockouts measured by the tail suspension test. Time spent immobile for the duration of the tail suspension test was measured in male (squares) and female (circles) clock gene knockouts. Reduced immobility time was observed in male (A n = 7-9) *Bmal1*-NAcKO compared to controls, but not females (B n = 8-9). No differences in immobility time were observed between male (C n = 12-13) *Per2*-NAcKO and controls (D n = 12-13). Higher immobility time resulted by the female (D n = 7-9) *Per2*-NAcKO. Results are depicted as mean ± standard error of the mean. *…$p < 0.05$. Details of independent measures t-test summarized in Table 1.

Table 1. *Behavioral consequences in males and females with NAc clock gene knockout*

		Males	Females
Behavioral test	**Clock gene**	**NAcKO vs NAcCNTRL**	**NAcKO vs NAcCNTRL**
EPM Open arm time	*Bmal1*	$t(14) = 0.96$; $p = 0.35$; $\eta^2 = 0.06$	$t(15) = 1.33$; $p = 0.20$; $\eta^2 = 0.11$
	Per2	$t(12) = 0.78$; $p = 0.45$; $\eta^2 = 0.05$	$t(14) = 2.80$; $\mathbf{p = 0.01}$; $\eta^2 = 0.36$
EPM Open arm entries	*Bmal1*	$t(14) = 1.08$; $p = 0.30$; $\eta^2 = 0.08$	$t(15) = 0.92$; $p = 0.37$; $\eta^2 = 0.05$
	Per2	$t(12) = 0.10$; $p = 0.93$; $\eta^2 < 0.01$	$t(14) = 2.57$; $p = \mathbf{0.02}$; $\eta^2 = 0.32$
EPM distance travelled	*Bmal1*	$t(14) = 1.16$; $p = 0.27$; $\eta^2 = 0.09$	$t(15) = 0.50$; $p = 0.63$; $\eta^2 = 0.02$
	Per2	$t(12) = 0.71$; $p = 0.49$; $\eta^2 = 0.04$	$t(14) = 1.72$; $p = 0.11$; $\eta^2 = 0.17$
OFT Latency to enter center	*Bmal1*	$t(14) = 1.64$; $p = 0.12$; $\eta^2 = 0.16$	$t(15) = 1.85$; $p = 0.08$; $\eta^2 = 0.19$
	Per2	$t(23) = 1.58$; $p = 0.13$; $\eta^2 = 0.10$	$t(14) = 2.25$; $\mathbf{p = 0.04}$; $\eta^2 = 0.27$
OFT % time in center	*Bmal1*	$t(14) = 0.41$; $p = 0.69$; $\eta^2 < 0.01$	$t(15) = 0.87$; $p = 0.40$; $\eta^2 = 0.05$
	Per2	$t(23) = 0.29$; $p = 0.77$; $\eta^2 < 0.01$	$t(14) = 1.34$; $p = 0.95$; $\eta^2 = 0.2$
OFT Distance travelled	*Bmal1*	$t(14) = 1.16$; $p = 0.27$; $\eta^2 = 0.09$	$t(15) = 0.06$; $p = 0.95$; $\eta^2 < 0.01$
	Per2	$t(23) = 0.63$; $p = 0.53$; $\eta^2 = 0.02$	$t(14) = 0.93$; $p = 0.37$; $\eta^2 = 0.06$
MBT % marbles buried	*Bmal1*	$t(14) = 0.41$; $p = 0.69$; $\eta^2 = 0.01$	$t(15) = 0.69$; $p = 0.50$; $\eta^2 = 0.03$
	Per2	$t(23) = 1.43$; $p = 0.17$; $\eta^2 = 0.08$	$t(14) = 0.43$; $p = 0.67$; $\eta^2 = 0.01$
TST % time immobile	*Bmal1*	$t(14) = 2.23$; $\mathbf{p = 0.04}$; $\eta^2 = 0.26$	$t(15) = 1.44$; $p = 0.17$; $\eta^2 = 0.12$
	Per2	$t(23) = 1.45$; $p = 0.15$; $\eta^2 = 0.08$	$t(14) = 3.12$; $\mathbf{p = 0.01}$; $\eta^2 = 0.41$

Note. Computed using alpha .05. Bolded *p* values highlight significant results.

Voluntary ethanol intake of *Bmal1* and *Per2* nucleus accumbens knockout mice

To assess the role of clock gene knockout in the NAc on voluntary ethanol consumption in male and female mice, an 11-session intermittent access to ethanol with two bottle choice paradigm was used. A 2-way repeated measures analysis of variance (RM-ANOVA) using session (i.e. 11 sessions) and virus (i.e., NAcCNTRL vs NAcKO) was conducted. Detailed statistics are depicted on Table 2. The RM-ANOVA revealed a main effect of the *Bmal1* NAc knockout on ethanol consumption and preference in males and females (Figure 6). On average, male *Bmal1*-NAcKO consumed 4 g/kg more ethanol and showed 21% higher ethanol preference compared to controls (Figure 8 A-D). Similarly, female *Bmal1*-NAcKO drank more than controls by an average of 5 g/kg, and preferred ethanol over water 18% more than *Bmal1* control females (Figure 8 E-H). In addition, the RM-ANOVA revealed a significant effect of session in both male and female *Bmal1* knockout (Table 2). By visual inspection of Figure 6 E and G, female *Bmal1*-NAcKO ethanol intake and preference escalated over the 11-sessions whereas female *Bmal1*-NAcCNTRL consumption remained stable over time. This observation is supported by the significant interaction effect of session and virus in female *Bmal1* ethanol intake and preference (Table 2).

Per2 deletion within the NAc of male mice also affected voluntary ethanol consumption and preference. Male *Per2*-NAcKO consumed more ethanol and exhibited higher ethanol preference than controls (Figure 9 A-D). On average, male *Per2*-NAcKO drank 3 g/kg more ethanol and displayed 12% higher ethanol preference than controls. In contrast to males, in females, *Per2* ablation in the NAc did not affect ethanol drinking behavior (Figure 9 E-H). Finally, no change in body weights were found in any clock gene knockout group across the 11-sessions (Figure 10). Detailed statistics are reported in Table 2.

Sucrose consumption of *Bmal1* and *Per2* nucleus accumbens knockout

Kampov-Polevoy et al. (1999) has suggested that mice have a propensity to consume sweet solutions like sucrose and ethanol. To establish that changes in ethanol drinking and preference were not merely a result of ethanol's palatable tase, the sucrose preference test was conducted at the end of the intermittent access to ethanol experiment. Two tailed t-tests revealed no difference in voluntary intake of 0.25% sucrose solution in male or female clock gene knockouts (Figure 11).

Binge drinking behavior of *Bmal1*-NAcKO male and female mice

A two-way RM-ANOVA revealed a main effect of *Bmal1*-NAcKO on naïve male ethanol consumption in the drinking in the dark experiment (Table 2). Furthermore, Šidák's multiple comparisons test revealed a significant difference in ethanol consumed (M = 1.5 g/kg, CI [0.20, 2.0], $p < 0.05$) between knockout and control mice on session 4 (Figure 12 B). However, no differences in ethanol drinking were found in naïve female *Bmal1*-NAcKO.

Although pre-exposed male and female *Bmal1*-NAcKO mice consumed more ethanol than controls in the intermittent access paradigm, no significant differences in ethanol consumption were observed in the drinking in the dark test (Figure 12). Detailed statistics are summarized on Table 2.

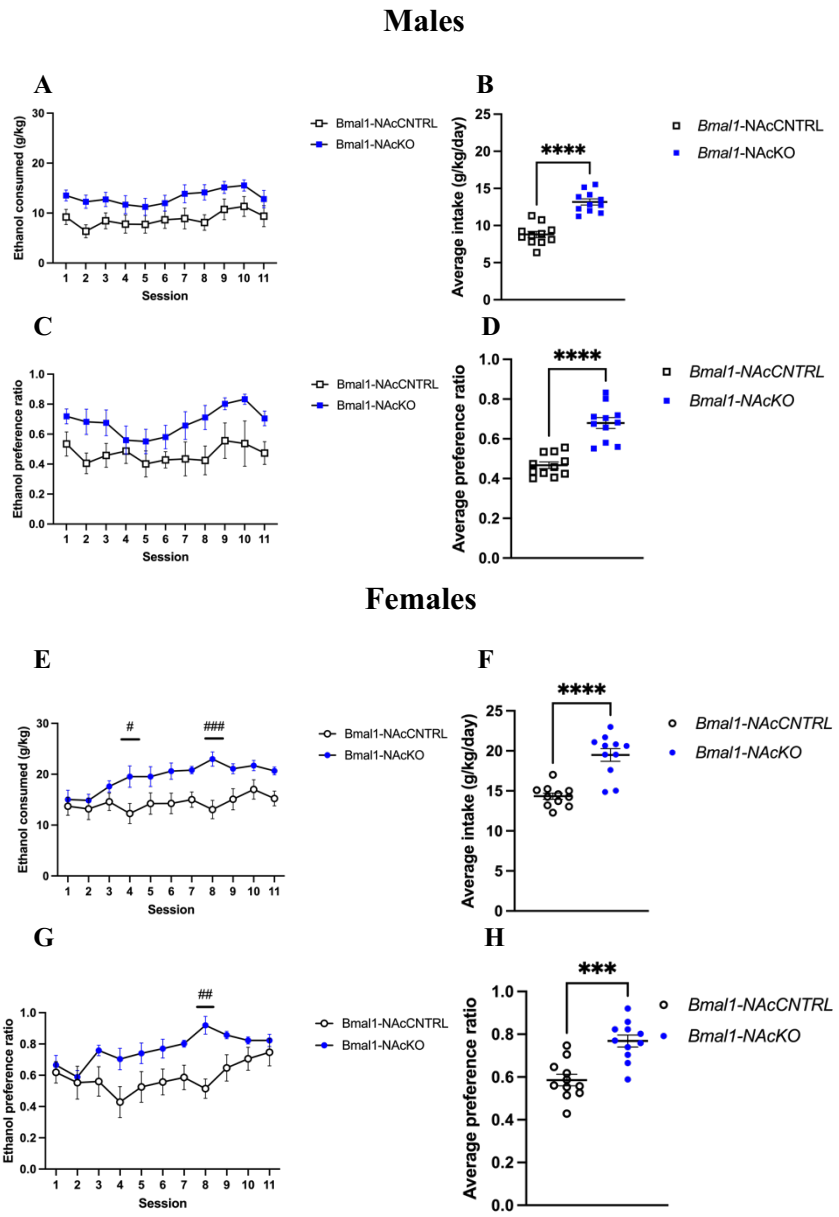

Figure 8. Voluntary ethanol intake of *Bmal1* nucleus accumbens knockout male and female mice.
Both males (A) and females (E) *Bmal1*-NAcKO consumed more ethanol than controls. Daily ethanol preference was higher in male (C) and female (G) *Bmal1*-NAcKO than controls. Average ethanol intake and preference per session are depicted for males (B, D) and females (F, H). Values are depicted as mean ± standard error of the mean. *… $p < 0.05$, **…$p < 0.005$, ***… $p < 0.0005$, **** … $p < 0.0001$. Šidák's multiple comparisons test: #… $p < 0.05$, ##…$p < 0.005$, ###… $p < 0.0005$. Statistical results are summarized in Table 2 and 3.

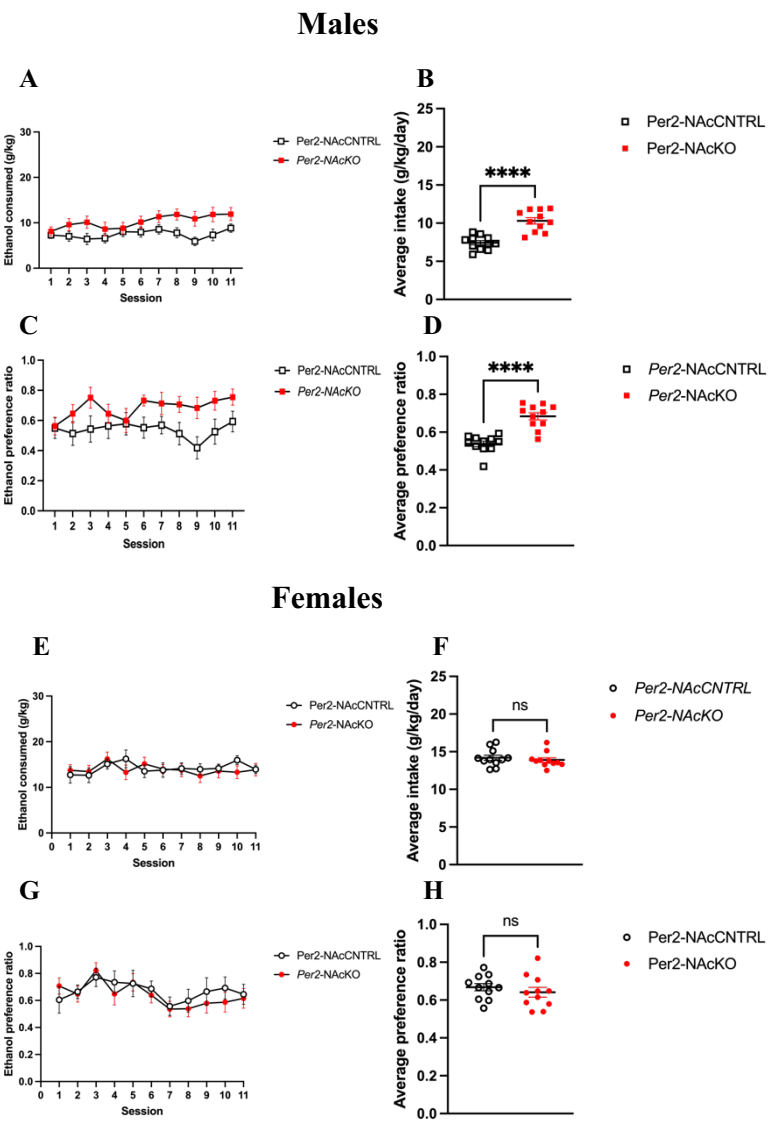

Figure 9. Voluntary ethanol intake of *Per2* nucleus accumbens knockout male and female mice. Males *Per2*-NAcKO consumed more ethanol (A) and displayed higher ethanol preference (C) than controls. No differences in ethanol consumption or preference were observed between female *Per2*-NAcKO and controls (E, G). Average ethanol intake and preference per session are depicted for males (B-D) and females (F-H). Values are depicted as mean ± standard error of the mean. *… $p < 0.05$, **…$p < 0.005$, ***… $p < 0.0005$, **** … $p < 0.0001$. Statistical results are summarized in Table 2 and 3.

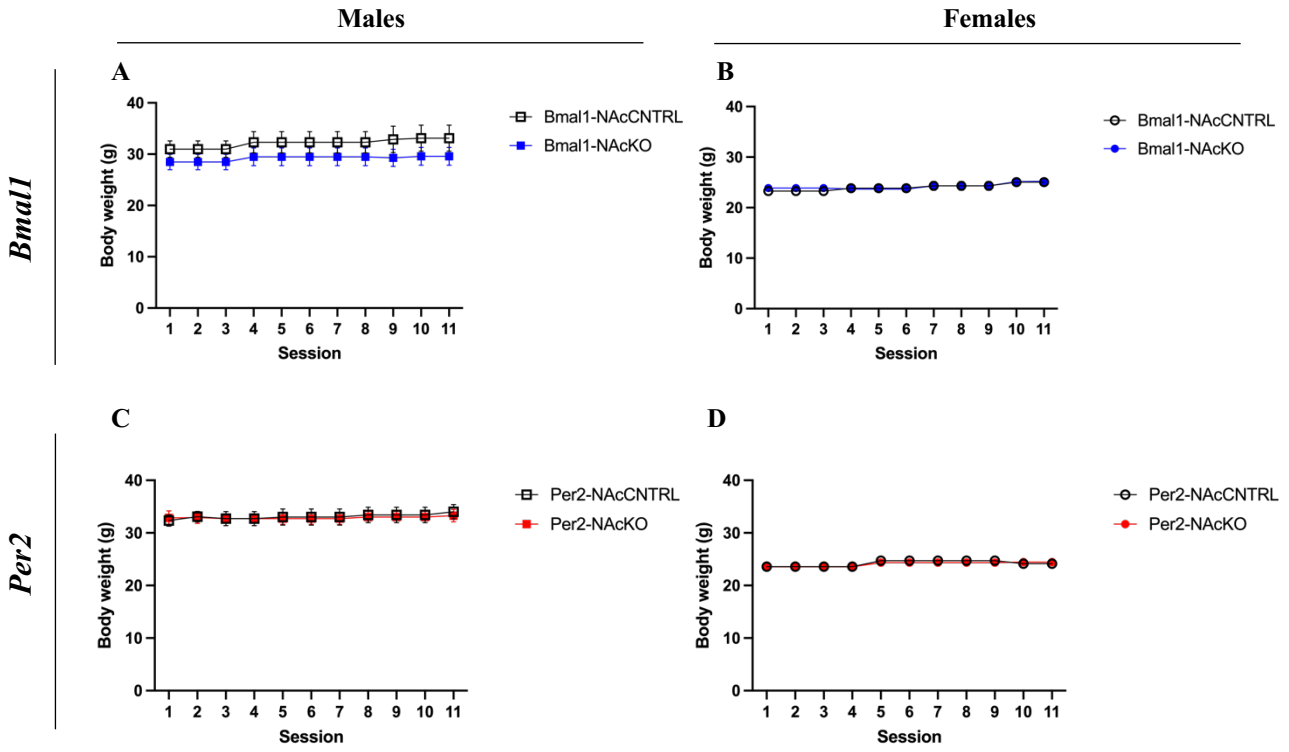

Figure 10. Body weights of clock gene knockout during intermittent access to ethanol.
Neither male *Bmal1*-NAcKO (A) or female *Bmal1*-NAcKO (B) mice differ in body weights across the 11 sessions of intermittent access to ethanol, compared to controls. No body weight differences were observed in either male (C) *Per2*-NAcKO or female (D) *Per2*-NAcKO and controls, during the intermittent access to ethanol paradigm.

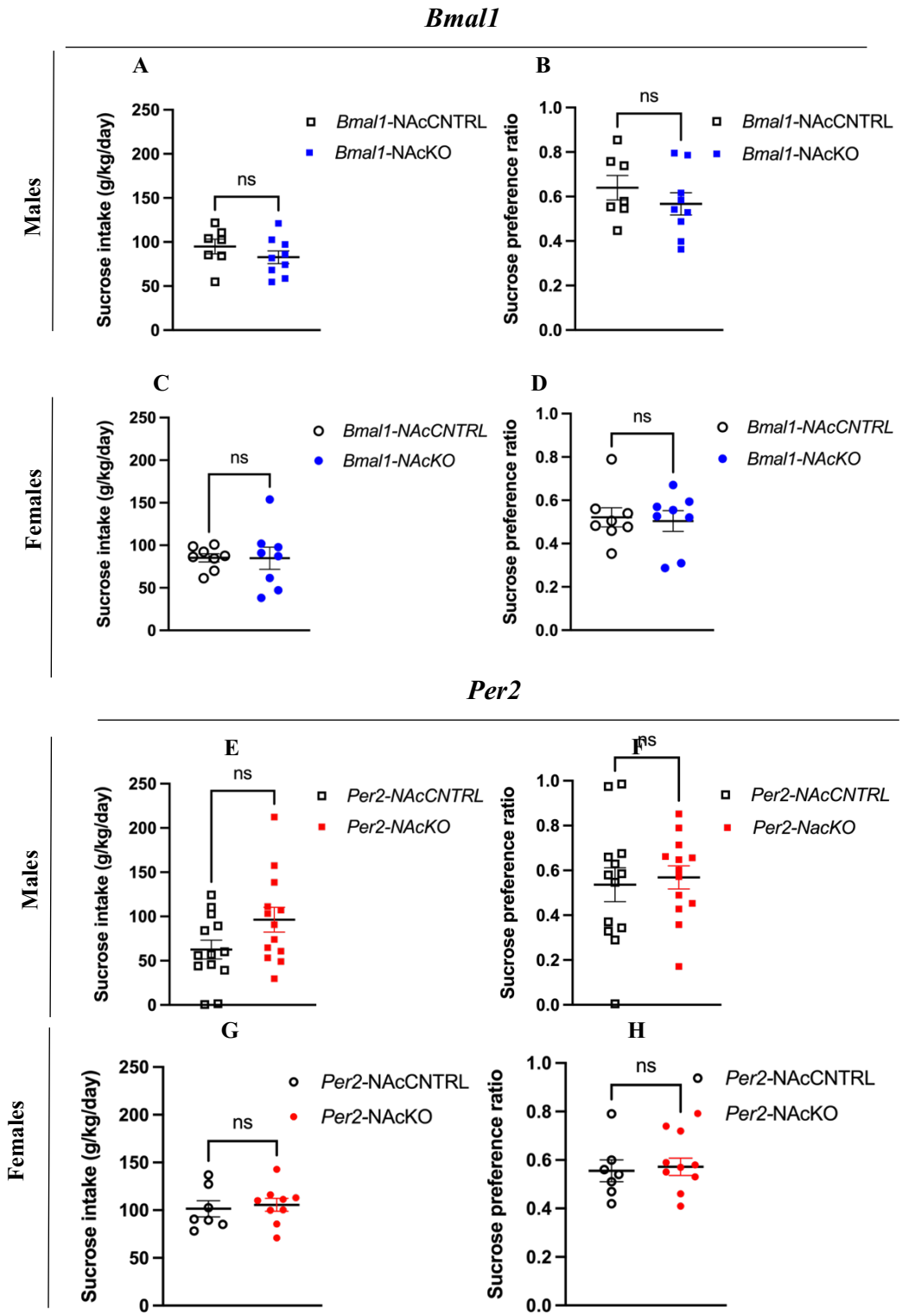

Figure 11. Voluntary sucrose intake of *Bmal1* and *Per2* nucleus accumbens knockout in male and female mice. No differences in sucrose intake were observed in either male (A), female (C) *Bmal1*-NAcKO or male (E), female (G) *Per2*-NAcKO. Similarly, sucrose preference ratios reveal no significant differences between clock gene knockouts and controls (B, D, F, G). Values are depicted as mean ± standard error of the mean. Statistical results are summarized in Table 3.

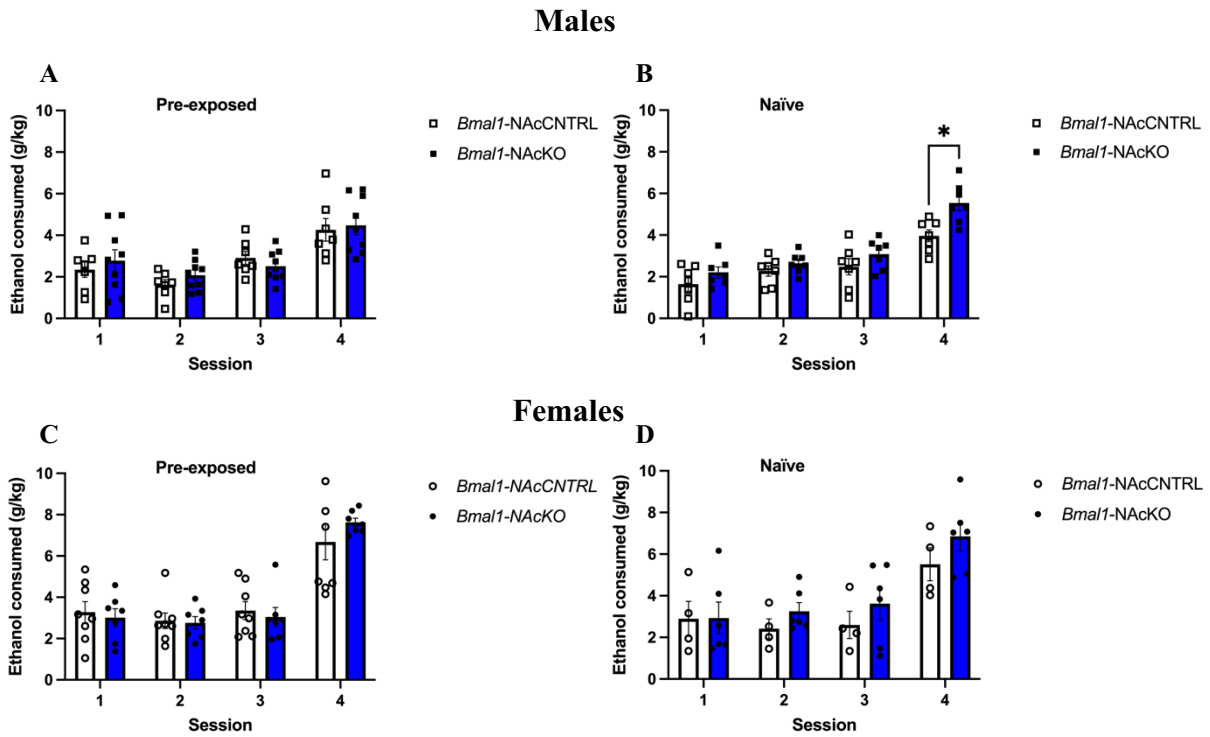

Figure 12. *Bmal1* nucleus accumbens knockout binge drinking behavior.
Males with *Bmal1* nucleus accumbens knockout that were naïve to ethanol (B) consumed more 20%(v/v) ehtanol in in the drinking in the dark test compared to controls, especially during session 4. Prior exposure to ethanol (A) did not affect binge drinking behavior is Male *Bmal1*-NAcKO. Similarly, no difference in binge drinking behavior was found in female *Bmal1*-NAcKO either in the pre-exposed (C) or naïve (D) groups. Values are depicted as mean ± standard error of the mean. Šidák's multiple comparisons test: *… $p < 0.05$. Statistical results are summarized in Table 2.

Table 2.
Ethanol drinking behavior in males and females with nucleus accumbens clock gene knockout

Drinking paradigm/Gene		**Two-way repeated measures ANOVA factors**		
Intermittent Access	**Sex**	**Session**	**Virus**	**Interaction**
Consumption *Bmal1*	Males	$F(5,66) = 2.94$; $p = 0.02$	$F(1, 14) = 5.91$; $p = 0.03$	n.s.
Preference *Bmal1*		n.s.	$F(1, 14) = 8.44$; $p = 0.01$	n.s.
Consumption *Bmal1*	Females	$F(10,150) = 3.84$; $p < 0.01$	$F(1, 15) = 8.40$; $p = 0.01$	$F(10, 150) = 2.16$; $p = 0.02$
Preference *Bmal1*		$F(5, 69) = 4.10$; $p = 0.02$	$F(1,15) = 5.89$; $p = 0.03$	$F(10, 150) = 2.24$; $p = 0.02$
Consumption *Per2*	Males	$F(6, 141) = 2.74$; $p = 0.01$	$F(1, 23) = 4.64$; $p = 0.04$	n.s.
Preference *Per2*		n.s.	$F(1, 23) = 4.33$; $p = 0.05$	n.s.
Consumption *Per2*	Females	n.s.	n.s.	n.s.
Preference *Per2*		$F(5, 65) = 6.38$; $p < 0.01$	n.s.	n.s.
Drinking in the dark				
Pre-exposed *Bmal1*	Males	$F(2,35) = 31.73$; $p < 0.01$	n.s.	n.s.
	Females	$F(3,39) = 47.78$; $p < 0.01$	n.s.	n.s.
Naïve *Bmal1*	Males	$F(2,24) = 47.47$; $p < 0.01$	$F(1, 12) = 7.11$; $p = 0.02$	n.s.
	Females	$F(2,17) = 27.48$; $p < 0.01$	n.s.	n.s.

Note. Computed using alpha .05. n.s. stands for not significant.

Table 3. *Average ethanol and sucrose intake in males and females with NAc clock gene knockout*

		Males	Females
Ethanol	**Clock gene**	**NAcKO vs NAcCNTRL**	**NAcKO vs NAcCNTRL**
Average intake per day	*Bmal1*	$t(20) = 7.36$, $p < 0.01$, $\eta^2 = 0.73$	$t(20) = 5.85$, $p < 0.01$, $\eta^2 = 0.63$
	Per2	$t(20) = 7.36$, $p < 0.01$, $\eta^2 = 0.73$	$t(20) = 0.66$, $p = 0.51$, $\eta^2 = 0.02$
Average preference per day	*Bmal1*	$t(20) = 6.62$, $p < 0.0001$, $\eta^2 = 0.67$	$t(20) = 4.69$, $p < 0.0001$, $\eta^2 = 0.52$
	Per2	$t(20) = 7.36$, $p < 0.01$, $\eta^2 = 0.73$	$t(20) = 0.82$, $p = 0.42$, $\eta^2 = 0.03$
Sucrose			
Average intake per day	*Bmal1*	$t(14) = 1.09$, $p = 0.29$, $\eta^2 = 0.13$	$t(14) = 0.35$, $p = 0.97$, $\eta^2 = 0.01$
	Per2	$t(24) = 1.91$, $p = 0.07$, $\eta^2 = 0.$	$t(14) = 0.39$, $p = 0.71$, $\eta^2 = 0.01$
Average preference per day	*Bmal1*	$t(14) = 0.97$, $p = 0.34$, $\eta^2 = 0.06$	$t(14) = 0.26$, $p = 0.80$ $\eta^2 = 0.005$
	Per2	$t(14) = 0.35$, $p = 0.73$, $\eta^2 = 0.01$	$t(14) = 0.29$, $p = 0.77$, $\eta^2 = 0.01$

Note. Computed using alpha .05

Discussion

This study examined the role of two core circadian clock genes within the NAc on affective-behaviors and ethanol consumption. We report that female *Per2* NAc knockout mice display differences in behaviors associated with anxiety and depression compared to controls. In addition, *Bmal1* NAc knockout reduces depressive-like states in males. No changes in mood behaviors resulted from the conditional knockout of *Bmal1* in females or NAc knockout of *Per2* in males. Regarding ethanol drinking behaviors, lack of *Bmal1* in the NAc caused an increase in ethanol consumption and preference in both males and females. *Per2* ablation in the NAc increased ethanol intake and preference in males but had no effect on consumption in females. No difference in sucrose preference was observed post ethanol exposure across all knockout groups. Binge drinking behavior was affected by *Bmal1* NAc knockout in males, which consumed greater amounts of ethanol during a four-hour period compared to controls, but this result was only found in mice without prior experience with ethanol. Taken together, these results suggest that abnormal *Bmal1* or *Per2* gene expression within the NAc cause mild changes to anxiety- and depressive-like behaviors but have significant effects on alcohol drinking.

Anxiety-like behavior

Anxiety-like behavior changes varied across experiment but were specific to female *Per2* NAc knockouts. In the elevated plus maze (EPM), deletion of *Per2* in females reduced the aversion to open spaces, suggesting that the deletion inhibited an anxiety-like response exclusively in females. In contrast, the absence of *Per2* increased the female latency to enter the center of an open field test (OFT), indicating an anxiogenic effect of the knockout. Anxiety-like behaviors are multifaceted and perhaps the EPM and OFT measure distinct aspects of the same condition (Lang and McTeague, 2009; Nakano et al., 2016; de Brouwer et al., 2019). Thus, NAc specific clock gene knockout might affect these behaviors differently. Although Russel et al. (2021) reported anxiolytic effects of global *Per2* knockouts in females evaluated in the O-maze, Spencer et al. (2013) suggested that knockdown of *Per2* in the male NAc causes anxiogenic effects in the EPM. Besides consistent use of behavioral assays, studies rarely include both male and female mice in their sample, therefore neglecting possible sex differences in behavior (An et al., 2011; Ngun et al., 2011), like the ones we have found, and making it difficult to gather a conclusive picture of how genetic manipulations affect anxiety-like behaviors. Recent work on male and female *Per2* deletion in whole striatum medium spiny neurons (MSN) revealed no changes in anxious behaviors in the EPM or OFT (Schöttner et al., 2022). Also, Martini et al.

(2021) reported no differences in anxiety-like behavior in males with glial *Per2* deletion within the NAc. Taken together, our results suggest that *Per2* regulates anxiety-like behavior either by dorsal striatal processes or by an astrocyte-dependent mechanism within the NAc.

Furthermore, this study did not find any difference in anxiety-like behaviors in *Bmal1* NAc knockout mice relative to controls. Although knockout of *Bmal1* in the limbic forebrain did not alter anxiety-like behavior (Snider et al., 2016), striatal *Bmal1* knockout had moderate anxiogenic effects in the EPM and OFT (Schöttner et al., 2022). Furthermore, Becker-Krail et al. (2022) reported *Bmal1* astrocyte knockout in the NAc increased anxiety-like behavior in the EPM. Thus, our study indicates that *Bmal1* influence on anxiety-like behavior is likely independent of neuronal NAc functions.

The mechanism by which *Per2* exerts its anxiolytic effects in the NAc remains elusive. Future research should address the relation between clock genes and sex differences within the ventral striatum, considering that estrogen receptors are shown to be clock controlled (Cai et al., 2008). Other clock genes should also be considered, since Ozburn et al. (2017) showed anxiolytic effects of *Npas2* knockdown within the NAc. Nonetheless, our results on anxiety-like behaviors support the notion that clock genes within the ventral striatum influence affective disorders.

Depressive-like behaviors

The conditional knockout of *Bmal1* reduced depressive like behaviors in males. Moreover, NAc deletion of *Per2* increased depressive-like phenotypes in females. Recent work on whole striatal *Bmal1* and *Per2* knockouts, using similar behavior paradigms, found reduced immobility in the TST of *Bmal1* knockouts, but no differences among sex (Schöttner et al., 2022). Taken together, these results indicate that depressive-like behaviors in male mice are influenced by a *Bmal1*-dependent mechanism within the dorsal striatum. In contrast, depressive-like behavior in female mice involves *Per2* NAc specific processes. Hampp et al. (2008) has suggested that *Per2* knockout deregulates monoamine oxidase-a (*Maoa*) expression, in both the dorsal and the ventral striatum, which results in a hyperdopaminergic state and therefore causes behavior alterations. More studies should be conducted to elucidate the interaction of clock genes within the NAc on dopamine signaling and behavior changes.

It should be noted that depressive-like behavior assessed in the TST was conducted at one time-point and it has been suggested that anhedonia, a type of depressive-like state, varies with

time of day (Liu et al., 2018). In that case, subsequent studies should consider running the TST at different time points to see if behavioral differences depend on the time of day.

Ethanol drinking behaviors

Previous work in our lab demonstrated a sexual dimorphic effect of striatal *Bmal1* knockout on ethanol consumption. Whereas male *Bmal1* striatal knockouts drank more ethanol, female mice drank less compared to controls (de Zavalia et al., 2021). Similarly, *Per2* striatal knockouts voluntarily drank more ethanol, but this effect was only found in males. The current study found similar augmented patterns of ethanol drinking behaviors in male *Bmal1* and *Per2* NAc knockouts. Strikingly, in contrast to the repressive effect of whole striatum *Bmal1* deletion on alcohol intake in females, selective deletion in the NAc augmented female alcohol consumption. This finding reveals that the female-specific inhibition of alcohol drinking in whole striatal *Bmal1* knockouts occurs outside of the NAc. Therefore, *Bmal1* deletion has both an inhibitory effect on female ethanol consumption, mediated by either the dorsal medial (DMS) or dorsal lateral (DLS) striatum, and a stimulatory effect mediated by a NAc specific mechanism. No difference in body weight or sucrose preference was observed, indicating that changes in ethanol consumption are not due to changes in body mass or sweet solution taste.

Studies on wild type rodents suggest that acute alcohol consumption corresponds to the hedonic effects processed by the NAc while long-term alcohol consumption is attributed to habitual behavior controlled by the DLS (Lagström et al., 2019; Corbit et al., 2012; Depoy et al., 2013). In our study we found that naïve male *Bmal1* NAc knockouts consumed more ethanol than controls in the drinking in the dark test (DID, an acute exposure to ethanol), while no difference in consumption was observed in the pre-exposed group. Therefore, it is tempting to speculate that the *Bmal1*-mechanism in the NAc is mediating reward-based alcohol drinking behavior. Compared to controls, there were no changes in ethanol binge drinking in female *Bmal1* knockouts regardless of prior experience with ethanol, indicating that binge drinking in females does not involve a *Bmal1* mechanism in the NAc.

In general, relatively little is known about the mechanisms by which clock genes affect ethanol consumption. Most often alcohol use disorders are attributed to dysfunctional glutamatergic neurotransmission (Tsai et al., 1998; Siggins et al., 2003). In turn, pharmacological treatments for alcoholism commonly target the glutamatergic system (Murphy et al., 2021; Carmen et al., 2004). In rodents, ethanol dependent mice displayed a twofold increase of

extracellular glutamate within the NAc (Griffin et al., 2014). Spanagel et al. (2005) linked higher ethanol intake in *Per2* knockout mice to a hyper-glutamatergic state. It's tempting to speculate that our ethanol-phenotypes are due to clock-gene induced changes of glutamate function, however, neither whole striatal (de Zavalia et al., 2021) nor NAc specific knockout of *Per2* changed alcohol intake in female mice. Therefore, future studies should investigate the influence of *Bmal1* and *Per2* on glutamatergic signaling within the NAc in a sex-specific manner within the context of alcohol consumption.

Limitations

The NAc is heavily populated by medium spiny neurons that express either Dopamine 1 (D1) or Dopamine 2 (D2) receptors. It has been shown that D1 and D2 receptor agonists cause different expression of clock genes in MSNs (Imbesi et al., 2009). Furthermore, studies show that D1 and D2 receptor-bearing MSNs play opposing roles in behavior (Kravitz et al., 2010, 2012; Durieux et al., 2013). In relation to ethanol consumption, for example, Cheng et al. (2017) demonstrate the mechanism by which D1 and D2 cells contribute to ethanol intake, such that stimulation of D1 cells increases ethanol intake while D2 cells suppresses the behavior. In addition, NAc also contains astrocytes (Scofield and Kalivas, 2014). Recent work from Becker-Krail et al. (2022) demonstrated the role of circadian clock genes in astrocytes of the NAc and its effects on behavior as well as neurotransmitter signaling. Therefore, variations in mood and ethanol consumption might be explained not only in a region-specific manner, like we show in this study, but also in a cell type specific way. Future studies should use cell-type specific viruses to assess behavioral disturbances of clock gene knockouts within the NAc.

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