

UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Role of Circadian Rhythms in the Development of Depression -like Behavior in Mice

Permalink

<https://escholarship.org/uc/item/499259vk>

Author

Long, Jaimie Elizabeth

Publication Date

2015

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Role of Circadian Rhythms in the Development of Depression-like Behavior in Mice

A Thesis submitted in partial satisfaction of the requirements for the degree Master of
Science

in

Biology

by

Jaimie Elizabeth Long

Committee in charge:

Professor David Welsh, Chair
Professor Nicholas Spitzer, Co-Chair
Professor Susan Golden

2015

The Thesis of Jaimie Elizabeth Long is approved and it is acceptable in quality and
form for publication on microfilm:

Co-Chair

Chair

University of California, San Diego

2015

DEDICATION

I dedicate this Thesis to my parents Libby and Steve for their love and support.

TABLE OF CONTENTS

Signature Page.....	iii
Dedication.....	iv
Table of Contents.....	v
List of Figures.....	vi
List of Tables.....	vii
Acknowledgements.....	viii
Abstract of the Thesis.....	ix
Introduction.....	1
Materials and Methods.....	8
Results.....	18
Discussion.....	37
References.....	42

LIST OF FIGURES

Figure 1: AAV induced Bmal1 knockdown reliably suppresses rhythmic PER2::LUC expression in SCN in vivo.....	10
Figure 2: Behavioral test time course.....	11
Figure 3: LH, TST and SP results from mice with disrupted rhythms in the NAc....	21
Figure 4: Open Field Test results from mice with BMAL1 knockdown in the NAc.	22
Figure 5: LH, TST and SP in mice with AAV injections in the PAG.....	25
Figure 6: Open Field Test results from mice with BMAL1 knockdown in the PAG.	26
Figure 7: LH, TST, and SP results in mice with AAV injections in the DR.....	29
Figure 8: Open Field Test results from mice with BMAL1 knockdown in the DR...	30
Figure 9: PER2 rhythm amplitudes from SCN explants of BMAL1-knockdown injected mice are significantly lower than controls.....	31
Figure 10: LH results from mice injected with AAV in the SCN.....	33
Figure 11: SCN BMAL1 knockdown mice performance in tail suspension and sucrose preference.....	34
Figure 12: SCN BMAL1 knockdown Open Field Test results.....	35

LIST OF TABLES

Table 1: Serotonin Receptor DNA Primers.....	17
--	----

ACKNOWLEDGEMENTS

I would like to thank Dr. David Welsh for welcoming me into his lab and helping me learn about circadian rhythms and psychiatry. I could not have completed my research without his support and guidance as my advisor.

I would also like to thank Dr. Dominic Landgraf for being very knowledgeable and a helpful supervisor throughout my research. I learned many invaluable research techniques, practices, and information about circadian rhythms from his expertise. Thank you as well for helping edit this thesis.

In addition, I want to thank all members of the Welsh Lab for their support and advice during my time in the lab. Last, but not least, I'd like to acknowledge Dr. Susan Golden and Dr. Nicolas Spitzer for serving on my Thesis Committee.

ABSTRACT OF THE THESIS

Role of Circadian Rhythms in the Development of Depression-like Behavior in Mice

by

Jaimie Elizabeth Long

Master of Science in Biology

University of California, San Diego, 2015

Professor David Welsh, Chair

Mood disorders, like Major Depressive Disorder (MDD) and Bipolar Disorder (BD), are associated with circadian rhythm disruptions, but the causal relationship between the two is not well understood. Previous data from our lab show an association between depression-like helpless behavior in mice and loss of circadian rhythms in mood-regulating brain areas, including the nucleus accumbens (NAc) and the periaqueductal grey (PAG). These results led to the hypothesis that reduced circadian rhythm amplitude in mood-regulating brain areas contributes to the

development of depression-like behaviors in mice, as tested by knockdown of an essential clock gene and several behavioral tests, including sucrose preference, open field test, tail suspension test, and learned helplessness. In addition, it is known that the PAG serotonin receptors (5HTR) play a critical role in developing helpless behavior. Hence, we hypothesized that circadian rhythms regulate 5HTR expression in the PAG and are involved in mood regulation. Our results indicate that losing rhythms in single mood-regulating brain regions is not sufficient for development of depression-like behavior in mice. However, disturbing rhythms throughout the entire brain by disrupting the central circadian pacemaker in the suprachiasmatic nucleus (SCN) leads to depression-like behavior in learned helplessness and significant differences in the open field test. These results suggest that disturbed circadian clocks may lead to the development of mood disorders and represent a starting point for other research to explain how circadian rhythm disruptions relate to mood disorders in psychiatric patients.

Introduction

Circadian Rhythms

The earth's rotations around its axis and the resulting daily environmental changes have caused most organisms to evolve circadian (24hr) rhythms. These rhythms alter an organism's activity levels, physiological processes, molecular expression, and behavior throughout the day to adapt to diurnal changes in the environment. In order to synchronize to environmental changes, organisms process external cues about environmental changes, called zeitgebers (German, time giver). Environmental light is the main zeitgeber that regulates the timing of circadian rhythms in most organisms (Daan et al. 1976). In mammals, the suprachiasmatic nucleus (SCN) in the hypothalamus is the main pacemaker of the circadian system. The SCN sends synchronizing signals to the rest of the body, thus ensuring that all tissues are entrained to the external lighting conditions. The tissues use SCN input to regulate circadian rhythmicity in their associated location; these secondary oscillators are referred to as peripheral clocks (peripheral meaning outside of the SCN) (McCarthy et al. 2012). The peripheral clocks control fundamental processes like gene expression, activity levels, digestion, heart rate, hormone levels, and body temperature (Kronfeld-Schor et al. 2012).

Regulation of Mammalian Circadian Rhythms

The SCN is located in the hypothalamus above the optic chiasm (crossing point of the optic nerves), where it receives direct environmental light information via the retinohypothalamic tract (Dibner et al. 2010). Each SCN nucleus consists of about

10,000 neuronal cells (Welsh et al. 2010). The retino-hypothalamic tract allows light information from retinal ganglion cells in the eye to project to the hypothalamus, thus reaching the SCN. (Welsh et al. 2010). This processing occurs because retinal ganglion cells in the eye contain a photo-pigment called melanopsin that allows them to be intrinsically photosensitive and respond to changes in light conditions; they are often referred to as intrinsically photosensitive retinal ganglion cells (ipRGCs) (Güler et al. 2008, Schmidt et al. 2011). Therefore, light information taken directly from the environment projects to the SCN, and the SCN can then use this information to remain synchronized to the light-dark cycle and project this synchronization to other parts of the body (Schmidt et al. 2011). When the SCN becomes arrhythmic or absent, it is unable to send synchronizing signals to other cells in the body. Those individual cells will then start to desynchronize from each other because each cell has a slightly different rhythm phase and period (Welsh et al. 2010). Therefore, the SCN is a very important brain region for regulating circadian rhythmicity and acting as the pacemaker of the circadian system.

Molecular Circadian Oscillations

Circadian rhythmicity in all mammalian cells is controlled by a molecular transcriptional-translational negative feedback loop (TTL) that takes about 24 hours to complete (Partch et al. 2014). This feedback loop is complex, but the main pathway consists of two transcriptional activators and two transcriptional repressors. In the morning hours, the transcriptional activators CLOCK and BMAL1 bind to form a heterodimer (Silver et al. 2014). The CLOCK-BMAL1 complex binds to an E-box

region in promoters to activate transcription of repressor genes *Period* (*Per 1, 2, 3*) and *Cryptochrome* (*Cry 1, 2*) (Silver et al. 2014). Later in the day the PER and CRY proteins get located back into the nucleus where they form a heterodimer to repress CLOCK-BMAL1 activation of *Per* and *Cry* gene transcription (Ye et al. 2014). Thus, accumulation of PER and CRY proteins in the cell nucleus causes a negative feedback loop to inhibit its own transcription. Later, this inhibition is lost because PER and CRY proteins get phosphorylated and targeted for degradation, thus allowing the cycle to start over (Silver et al. 2014). This basic molecular process is what drives circadian rhythmicity in all mammals.

The Role of Circadian Rhythms in Mood Disorders

Circadian rhythms are thought to play a role in psychiatric mood disorders since they regulate physiology and behavior. For instance, many mood disorder patients display disruptions of circadian processes, such as sleeping, eating, and hormone cycles (McCarthy et al. 2012). These mood disorders include major depressive disorder (MDD), seasonal affective disorder (SAD), anxiety, and bipolar disorder (McCarthy et al. 2012; Wulff et al. 2010). In order to decipher how circadian rhythms and psychiatric disorders are related, researchers initially looked to the SCN as a possible target (McCarthy et al. 2012). However, there has been a recent focus on non-SCN mood-regulating brain areas as a likely contributor to mood disorder development (McCarthy et al. 2012). Research in this field is crucial to understanding how mood disorders develop and for creating more effective and reliable treatments against these disorders.

Despite the clinical connection between circadian rhythms and mood disorders, current difficulties in studying circadian rhythms in mood disorders have hindered research discoveries in this topic. Currently, a major question that is yet to be solved is whether a disruption in circadian rhythms can exclusively cause mood disorders (Landgraf et al. 2014). This question has proved difficult to test in living human patients because researchers need brain tissue to determine rhythmicity of brain areas. Therefore, animal models have been developed to determine causality between circadian rhythms and mood disorders (Landgraf et al. 2014). For example, many clock gene mutations in mice have been shown to produce different behavioral phenotypes associated with mood disorders. Unfortunately, it is unclear whether these phenotypes are exclusively caused by disturbance of circadian rhythms or other non-circadian factors, since some genes involved in the circadian TTL also regulate non-circadian processes (Landgraf et al. 2014). In addition, it is also possible that circadian rhythmicity and mood disorders are affected by a common physiological process, but do not affect each other directly (Landgraf et al. 2014).

Regardless of the challenges in studying circadian rhythms and mood disorders, several important research studies have shown a strong connection between mood disorders and circadian rhythms. One of these was an extensive study that found disruption in circadian gene expression from mood-associated brain tissue of postmortem MDD patients compared to control brains (Li et al. 2013). Patients' gene expression was measured at the time of death and was combined and organized to span one circadian cycle. The findings revealed that circadian genes in mood-regulating brain areas had weaker rhythms than those of control subjects (Li et al.

2013). Even though this study used postmortem patients and a full rhythmic pattern for individual patients was not possible to obtain, this study was one of the first papers to confirm the possibility that circadian brain rhythms are disturbed in mood disorders in humans.

Other recent studies have used animal models to determine a causal relationship between circadian rhythms and mood disorders. One of these studies found that the circadian genes *Per1* and *Per2* in the brain region nucleus accumbens (NAc) are involved in the development of anxiety-related behavior (Spencer et al. 2012). The results indicated that a decrease in *mPer1* and *mPer2* expression, using a *Per1* and *Per2* knockdown, increases mice anxiety-related behavior in behavioral tests (Spencer et al. 2012). Although this finding suggests further evidence circadian rhythm disturbance can cause mood disorders, it does not show if lack of *Per1* and *Per2* changes the actual rhythmicity in the NAc, since *Per1* and *Per2* expression are only measured at one time point. This study coincides with unpublished data in our lab that shows a connection between depression-like behavior in mice and altered circadian rhythmicity in the NAc, Periaqueductal Grey (PAG), and the Dorsal Raphe Nucleus (DR).

The present study aims to further determine the connection between circadian rhythms and mood disorders and to help clarify the causality between them. Specifically, the two questions addressed in this thesis are (1) do disturbed circadian rhythms in specific mood-regulating brain areas contribute to the development of mood-related behavioral changes in mice, and (2) what are the physiological consequences of having disrupted circadian rhythms in mood-regulating brain regions,

and more precisely, how does it alter serotonin receptor (5-HTR) expression in the PAG?

To investigate the impact of disturbed circadian clocks in a subset of mood-regulating brain areas, I worked with mice that had locally disturbed circadian rhythms in the PAG, NAc, DR or SCN. Disturbance of these local rhythms is accomplished by a localized viral-mediated knockdown of BMAL1, an essential circadian clock gene. The virus is an shRNA AAV (Adeno-associated virus) that is targeted to degrade *Bmal1* mRNA, effectively creating a knockdown of BMAL1 protein. Knockdown of this gene disrupts the TTL feedback loop that drives circadian gene regulation and will cause localized arrhythmicity (Bunger et al. 2000). Control mice were injected with a scrambled sequence shRNA AAV without specific targets.

After BMAL1 knockdown, I characterized the mice's mood-related behavior with a focus on reward-, anxiety-, despair- and depression- like manners. I performed four behavioral tests on all of the mice: sucrose preference, open field test (OFT), tail suspension test (TST), and learned helplessness (LH). Differences in mood-related behaviors between the BMAL1 knockdown mice and the control mice provide insight on how the disturbance of circadian rhythms in certain brain areas affects mood disorders.

To answer the second question, rhythmic and arrhythmic cultured PAG explants were analyzed to determine the effect of circadian rhythms on local 5-HTR expression. 5-HTRs were chosen because serotonin has been implicated in mood disorders, and serotonin reuptake inhibitors are often used to treat MDD (Graeff et al. 1996). In addition, 5-HTRs in the PAG are known to play a role in the development of

helplessness (Graeff et al. 1996). To determine expression patterns of 5-HT_{1A} expression in rhythmic and arrhythmic PAG, the explants were collected at four time-points over 24 hours, and total RNA was isolated from these explants for use in qPCR to amplify 5-HT_{1A} expression. This experiment was designed to determine if there are physiological effects of having disrupted circadian rhythms in the PAG. Overall, this research provides preliminary work to determine if disrupted circadian rhythms in mood-regulating brain areas cause mice to develop depression-like behaviors, and could eventually be used to create novel treatments for mood disorder patients.

Materials and Methods

Animals

Eight-twelve week-old male mPer2Luciferase (PER2::LUC) mice were used for all studies. PER2::LUC mice are a strain in which the PER2 circadian protein is fused with luciferase. The luciferase gene is inserted into exon 23 of the *Per2* gene and does not affect endogenous rhythms (Yoo et al. 2004). Luciferase is an enzyme from the firefly that creates bioluminescence when chemically reacting with its substrate luciferin (Yoo et al. 2004). This mouse strain is useful because it allows recordings of real-time circadian rhythms of Per2 in cultured tissues and cells (Welsh et al. 2004).

Mice were single-housed under LD 12:12 (12hr light, 12hr dark, lights on at 06:00) conditions at all times. Five to six days before surgical injections, mice were separated from each other and single-housed because group housing has been shown to increase vulnerability in the LH paradigm due to altered stress-sensitivity (Chourbaji et al. 2005). Mice were randomly assigned to BMAL1-knockdown and control-injection groups and were kept in individually ventilated cages with bedding material and continuous access to water and food. Cages of mice used in the same experiment were kept immediately adjacent to each other on the rack in the housing room. Mice remained in the same cages throughout the whole experiment. The Institutional Animal Care and Use Committee at University of California, San Diego approved mouse studies. Every effort was made to minimize the number of animals used, and their suffering.

BMAL1 AAV brain injections

Dr. Dominic Landgraf performed all stereotactic AAV injections into specific brain areas. An AAV virus containing shRNA that targets *Bmal1* transcripts was used to eliminate BMAL1 protein production in locations where the virus was injected. As a control, a non-specific scrambled sequence AAV virus was injected using the same procedure. Dr. Landgraf injected the mice using a stereotactic apparatus to ensure precise localization of the AAV. The knockdown takes about 3 weeks to take full effect. AAV control and BMAL1 virus was injected into either the NAc, PAG, SCN or DR. A GFP reporter was part of the viral sequence to confirm that it was injected into the right brain areas. Figure 1A shows an example of AAV virus location using the GFP reporter in an SCN slice (right panel). BMAL1 knockdown efficiency in the SCN is demonstrated in figure 1B by a loss of PER2 rhythms measured by LumiCycle Analysis (bottom panel) compared to normal rhythms in the SCN with control scrambled sequence virus.

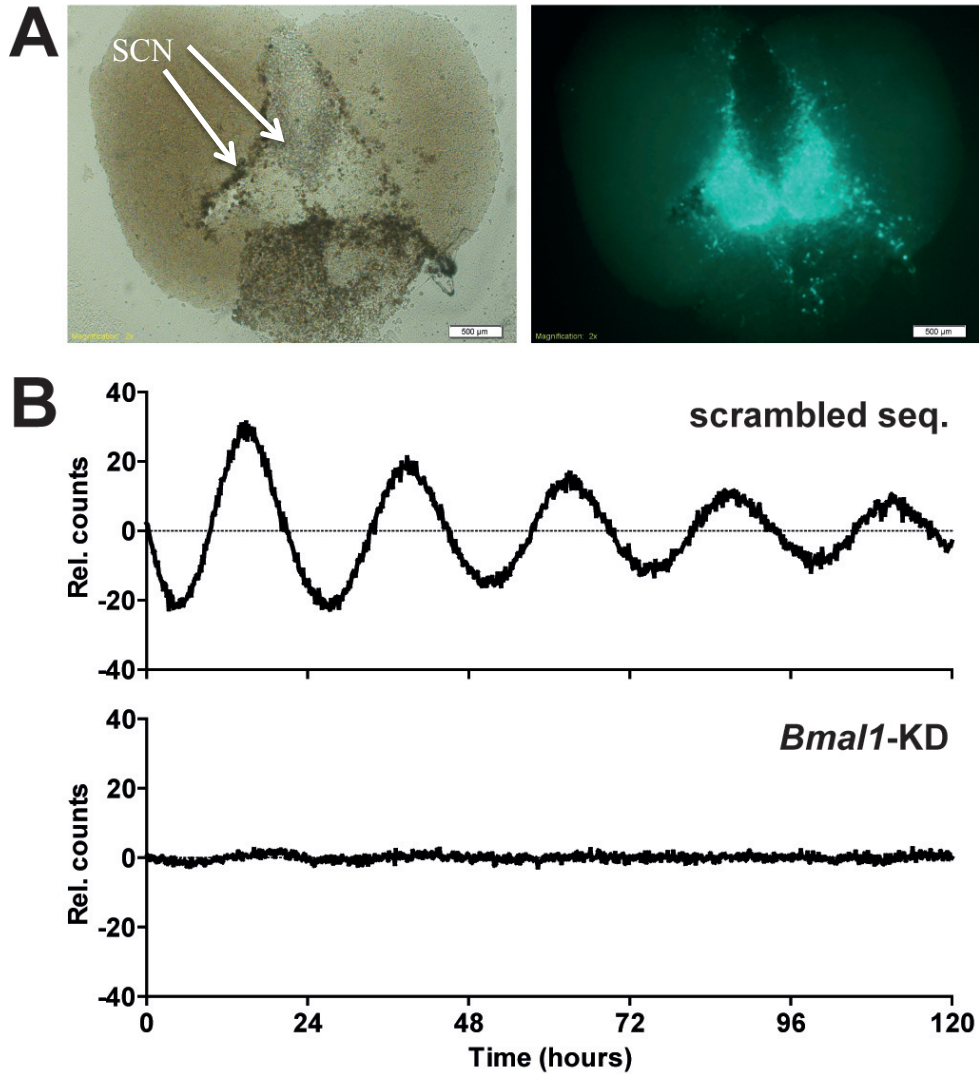


Figure 1: AAV induced Bmal1 knockdown reliably suppresses rhythmic PER2::LUC expression in SCN in vivo. (A) 5 weeks after injection of the AAV particles, brain slices of the SCN were prepared to confirm location of the injection site. Left: bright field image of organotypic SCN slice, right: AAV induced GFP expression in the SCN. (B) PER2::LUC expression of organotypic SCN slices 5 weeks after in vivo injection of control AAV particles (top) and of Bmal1-KD AAV particles (bottom).

Behavioral Tests Time Course

Sucrose preference was started for all animals before surgery. Initially, the time course of experiments was determined by the stress of the test so that less stressful tests were done before more stressful ones. This was done to test if

disturbance of rhythms leads to depression-like behavior before stress of learned helplessness. Therefore, open field test (OFT) was performed first, followed by tail suspension (TST) and then learned helplessness (LH) (Figure 2A). In other mice, LH was done a week before OFT and TST, to see if disturbance of rhythms makes mice more vulnerable to inescapable stress and catalyzes depression-like behavior (Fig. 2B). In addition, in some mice OFT and TST were done both before and after LH in order to observe within-subject differences (Fig. 2C).

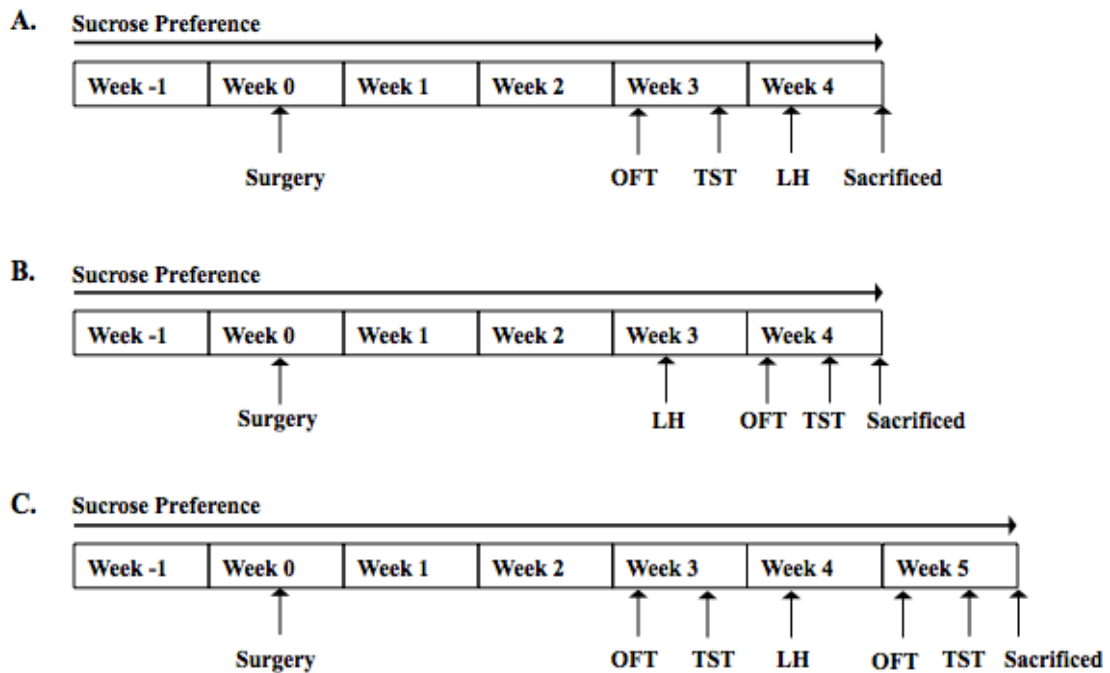


Figure 2: Behavioral test time course. For A, B, and C sucrose preference test was started when mice were placed in individual cages during week -1 and was continued throughout the whole experiment. Surgery to inject the virus was done at week 0, three weeks before the other behavioral tests were started. The order of OFT, TST, and LH changed based on the time course schedule, but the number of days between tests was always the same.

Sucrose Preference

Sucrose preference is a test used to determine reward-seeking behavior. After mice were transferred to single-housed cages, sucrose preference began. Water bottles were filled with 1% sucrose water and added to mouse cages in the food slot. Food was placed at the bottom of the cage. Sucrose and water bottles were weighed before being put into the cage. One day later, the sucrose and water bottles were weighed again to determine the amount of liquid consumed from each bottle. In order to avoid cage side preferences, placement of the sucrose water bottle was switched between the right and left side of the cage for each measurement. Sucrose preference was done two times per week until the mice were sacrificed. The measurement of sucrose preference compared the percentage of sucrose water the mouse consumed to the total volume of liquid consumed.

Open Field Test

Open field test (OFT) tracks a mouse's activity in an open box for a period of five minutes and is often used to determine anxious behavior and overall activity levels. Anxiety can be measured by analyzing freezing behavior and willingness to go into the unprotected center of the box. This behavioral experiment used equipment and software created by AccuScan Instruments Inc., to track mouse movements. OFT was performed at least 3 weeks after mice were injected with the AAV virus. On the day of testing, mice were taken to a dim-lit procedural testing room and placed in individual open field boxes. Mice were allowed to explore the box for 5 minutes while the software collected data on their movements every minute. After testing, the results

were analyzed for total distance moved, total time the mouse was immobile, total time spent in the center, latency to reach the center, distance moved in the center, and how long the mouse was standing on its hind legs (rearing).

Tail Suspension Test

Tail suspension is a test that measures a mouse's despair-like behavior when being suspended in the air, as described previously (Can et al. 2004). The test was originally designed to determine effectiveness of antidepressants, but is used here to determine despair-like behavior. The amount of immobility time is measured in this test to determine how long the mouse did not struggle and essentially gave up escaping. Greater immobility time is indicative of depression-like behavior. Tail suspension was performed 3 or 4 weeks after mice were injected with the AAV virus and 2-3 days after OFT. Mice were brought to a lab and allowed about 10 minutes to acclimate to the environment. A tail barrier fashioned out of a cut 50-ml plastic tube was placed around the base of the tail to prevent the mouse from climbing up its own tail during the procedure. Tape was cut to 17cm and one end was wrapped around the tip of the mouse's tail. The other end of the tape was stuck to a metal rod held up by a wooden frame with three walls to hold the mouse up by its tail. The walls on the wooden frame prevented the mice from seeing each other during the procedure. The mice were kept on the rod for 6 min in a hood while being videotaped. After 6 min, the mice were immediately taken off and placed back into their cages. The tape and cut tube were removed. The video was analyzed for each mouse to measure the amount of time the mouse was immobile and how long it took for the mouse to first

become immobile. Immobility was counted if the mouse stopped moving its hind legs, torso, and head for at least 2 sec.

Learned Helplessness

Refer to Dr. Landgraf's protocol for a detailed Learned Helplessness theory and procedure in mice (Landgraf et al. 2015). LH is a 3-day test used to measure a mouse's depression-like mood state after uncontrollable stress. On the first two days, called training, mice are given 120 inescapable tail shocks while restrained for one hour each day. Five second shocks were applied randomly every 20-30 sec to the mouse's tail through electrical wires. The intensity of the shocks started at 0.25mA and increased by 0.05 mA every 15 shocks to 0.60mA until 120 shocks were given. The shock intensity was increased so that the mice did not get habituated to the initial shock intensity. On the last day, called testing, mice were placed in a shuttle box, with two compartments connected by a gate, where 30 escapable foot shocks were administered (Landgraf et al. 2015). For the first minute of the test, mice were allowed time to acclimate to the box with the gate open. Then, a series of five 30-sec shocks occurred when the mouse could stop the shocks by crossing the gate one time (fixed ratio 1, FR-1). Next, 25 shocks were given for at most 30 sec and the mouse could stop the shocks early by crossing the gate two times (FR-2). This procedure demonstrates how hard the mouse will try to eliminate the shocks, and the mouse is thought to show depression-like behavior if it fails to make the effort to cross the gate two times to get rid of the shocks. From this test, the box's software measures the number of times the mouse failed to cross the gate to stop the shocks and the amount

of time it took for the mouse to escape the shocks (30 second latency means the mouse did not escape).

Mouse Brain Culture and Collection

After the behavioral tests, Dr. Landgraf sacrificed and removed the brains from PER2::LUC mice used. Slices were made of a certain brain area and incubated at 37°C in a Lumicycle that detects bioluminescence. In the Lumicycle, brain slices were cultured in a luciferin-rich medium on a Millicell Cell Culture Insert for 3-4 days until collection. Bioluminescence rhythms were monitored using Lumicycle analysis and the software determined the rhythm amplitude.

For PAG slices that were prepared for use in qPCR, determination of rhythmic and arrhythmic PAG slices was accomplished using the LumiCycle Analysis. If a slice was rhythmic, the phase and amplitude of PER2 rhythms was determined. PAG slices were taken out of the incubator at specific times in the circadian cycle to get a time course over a 24-hour period. Rhythmic slices were collected at CT 0, 6, 12, or 18 (CT= circadian time; CT 6 is the trough and CT 18 is the peak of the circadian cycle for Per2). Arrhythmic slices were collected every 6 hours at 00:00, 06:00, 12:00, or 18:00 o'clock. Brain slices were taken out of the incubator at the time of collection and removed from the membrane using PBS. The brain slice was then transferred to a 1.5 mL microfuge tube, excess PBS was removed, and the tube was placed in liquid nitrogen or dry ice. The slices were then stored in a -80°C freezer.

RNA Isolation

RNA isolation was achieved using QIAGEN RNeasy Micro kit and the associated protocol. View the QIAGEN RNeasy micro kit protocol for detailed information. Frozen brain slices were homogenized in the supplied buffer using a tissue disrupter for at least 1 min and pressed through an insulin syringe 5 times. The rest of the RNeasy Micro kit protocol was followed, excluding the DNase step. The resulting RNA was extracted from the column using 12 μ L RNase-free water. RNA concentration was measured for each sample using a NanoDrop spectrophotometer.

Reverse Transcription

After RNA was extracted from all of the PAG brain slices it was used in Reverse Transcription to make cDNA. For this experiment, a Reverse Transcription (RT) Kit from Biosystems was used. RNA was combined with RT buffer, Reverse Transcriptase, dNTPs, random primers, and RNase inhibitor from the kit. The samples were then placed in a thermal cycler programmed according to the kit's instructions. Each tube consisted of 5 μ L RNA sample and 5 μ L of RT master mix, for a total of 10 μ L per tube.

Quantitative PCR (qPCR)

The cDNA produced during Reverse Transcription was used in a qPCR experiment to detect 5-HTR RNA from mice PAG. For the qPCR, four 5-HTR primers were used along with a housekeeping gene primer for β -actin (Table 1). An initial PCR was done to create a Standard Curve of efficiency of the primers and quality of cDNA (Pfaffl, 2001). For this, 0.5 μ L cDNA from each sample was combined and diluted in

series with nuclease-free water in ratios of 1:10, 1:50, 1:250, 1:1250, and 1:1650. This was then added to each primer solution and SYBR-Green. Each combination of cDNA concentration and primer was replicated 3 times in the PCR plate to control for pipetting error. A negative control of just nuclease-free water and SYBR-Green was tested for each primer.

Then, another qPCR experiment was designed to test the expression of 5HTR in each PAG explant collected. For each time point, 5 PAG explants were collected that were either rhythmic or arrhythmic. The cDNA of each explant would be distributed in five wells individually, so that all four primers and the housekeeping gene could be tested separately for each cDNA sample. This was repeated with all cDNA samples at every time point.

Table 1: Serotonin Receptor DNA Primers. Four Serotonin receptor primers were created and used in qPCR. β -actin served as a housekeeping gene.

Gene	Right Primer	Left Primer
HTR1A	CCACTACCTGGCTGACCATT	GACGGTCAAGAAGGTGGAAA
HTR1F	TCAAGTTTTGGTCTGATGCG	AACAGTTGAGCCTGCCACAC
HTR2A	TCATCCTGTAGCCCGAAGAC	ATAGCCGCTTCAACTCCAGA
HTR2C	CAGACGGGGCACAAATATCTA	CATTGCTGATATGCTGGTGG
β-actin	CCCTGAAGTACCCCATTGAA	AGGTGTGGTGCCAGATCTTC

Results

Behavioral Tests

For all amplitude-correlated scatter plots, mice were categorized by BMAL1 knockdown injection (BMAL1) and the scrambled sequence control injection (control). Not all AAV BMAL1-injected mice showed a complete decrease in rhythm amplitude and some control-injected mice naturally had low rhythm amplitudes in some brain areas. Therefore, control and BMAL1 mice data were combined and correlated to rhythm amplitude (shown in blue). This was done to see if there was a correlation between rhythm robustness (regardless of injection) and differences in behavior. In analysis of sucrose preference, mice were grouped based on a low rhythm amplitude and a high rhythm amplitude.

Circadian Rhythm disruption in the NAc

All mice injected into the NAc were subjected to behavioral tests in the timeline described by Fig. 2A or 2B. AAV BMAL1-KD injections into the NAc were not always successful at reducing rhythmicity and rhythm amplitude, as seen by the wide spectrum of rhythm amplitudes for both BMAL1 and control mice (Fig. 3 and Fig. 4). It is likely that virus leaked into nearby ventricles during and after the injection. In addition, some control mice were naturally arrhythmic or had very low rhythm amplitudes. This variation in rhythmicity of the NAc was observed in previous NAc PER2 rhythm data from mice that did not have any injections. Therefore, all results compare the NAc rhythm amplitude to the behavioral test measurement.

Learned Helplessness

Independent of AAV injections, low circadian rhythm amplitude in the NAc had no significant impact on mice behavior in learned helplessness testing compared to controls (Fig. 3A). The average escape latency in LH testing did not correlate with circadian rhythm amplitude in the NAc (Fig. 3A, left panel). There was also no significant correlation between the number of escape failures during LH and the rhythm amplitude of the NAc (Fig. 3A, right panel).

Tail Suspension

Disturbed rhythms in the NAc had no significant impact on immobility time or immobility latency in TST before LH (Fig. 3B, left). After LH, disturbed rhythms in the NAc also did not significantly affect TST immobility time or immobility latency (Fig. 3B, right).

Sucrose Preference

Disrupted rhythms in the NAc (arrhythmic, with amplitude = 0) did not influence the mice's sucrose water preference compared to undisrupted rhythms (amplitude > 0). The percentage of sucrose water consumed over normal water throughout the experiment did not differ significantly between the arrhythmic and rhythmic groups as tested by two-way ANOVA (Fig. 3C).

Open Field Test

Disturbance of circadian rhythms in the NAc did not significantly affect mice performance in the OFT. In both before and after LH the total distance moved did not correlate with rhythm amplitude (Fig. 4A). Similarly, the immobility time before and after LH was not correlated with rhythm amplitude (Fig. 4B). The total time spent in the center before or after LH did not correlate with NAc rhythm amplitude (Fig. 4C). Mice were also tested for Center distance, Rear time, and Center latency, however these data were insignificant (data not shown).

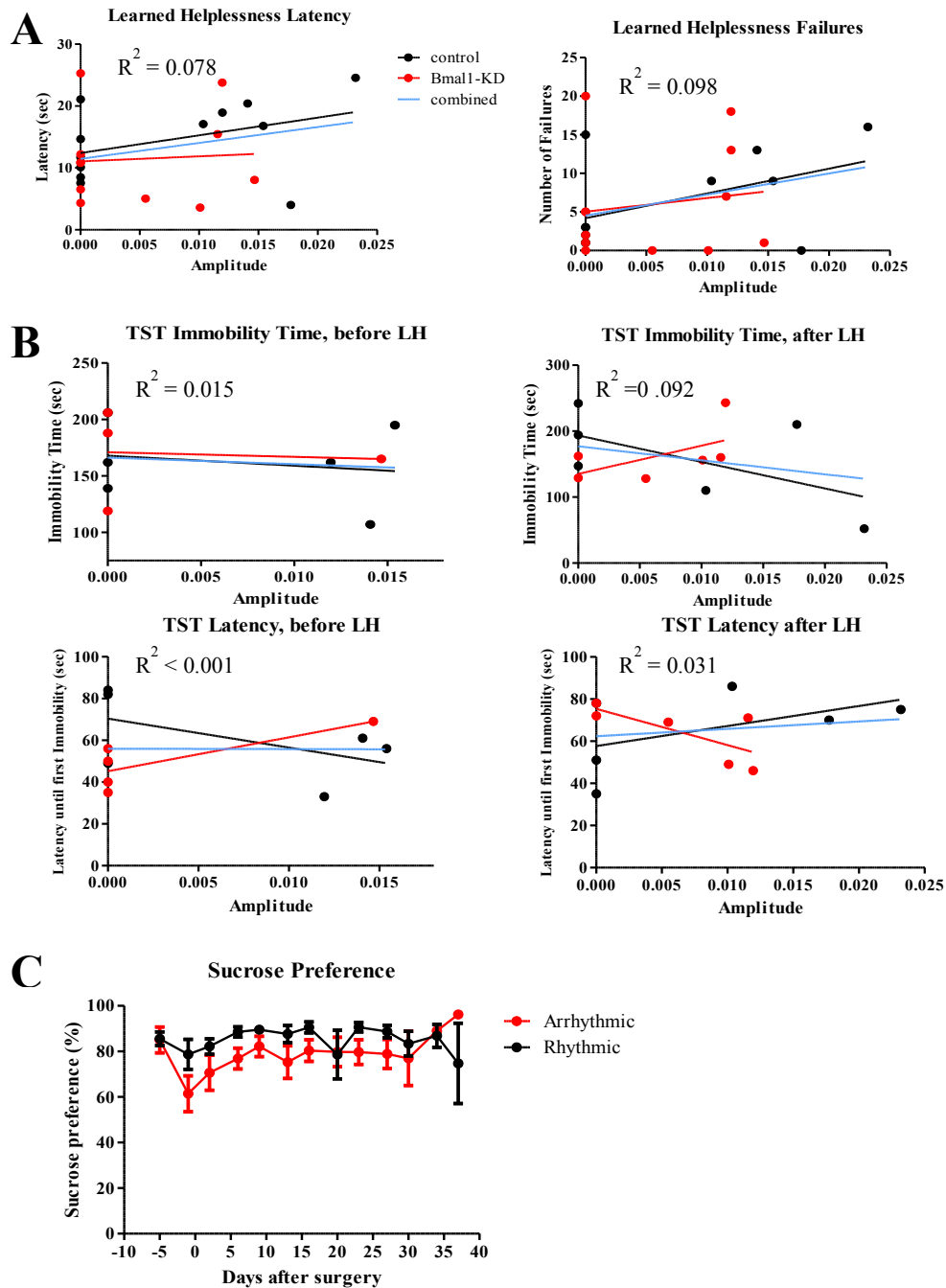


Figure 3: LH, TST and SP results from mice with disturbed rhythms in the NAc. A, LH escape latency and number of escape failures correlated to NAc rhythm amplitude; solid lines are linear regression lines, R^2 is for combined ($n=24$). B, TST immobility time and latency compared to rhythm amplitude before ($n=11$) and after LH ($n=12$); solid lines are linear regression lines, R^2 is for combined. C, Percentage of sucrose water consumed out of total liquid consumed for mice with rhythmic or arrhythmic NAc explants ($n=24$); data analyzed two-way ANOVA with Bonferroni post-test.

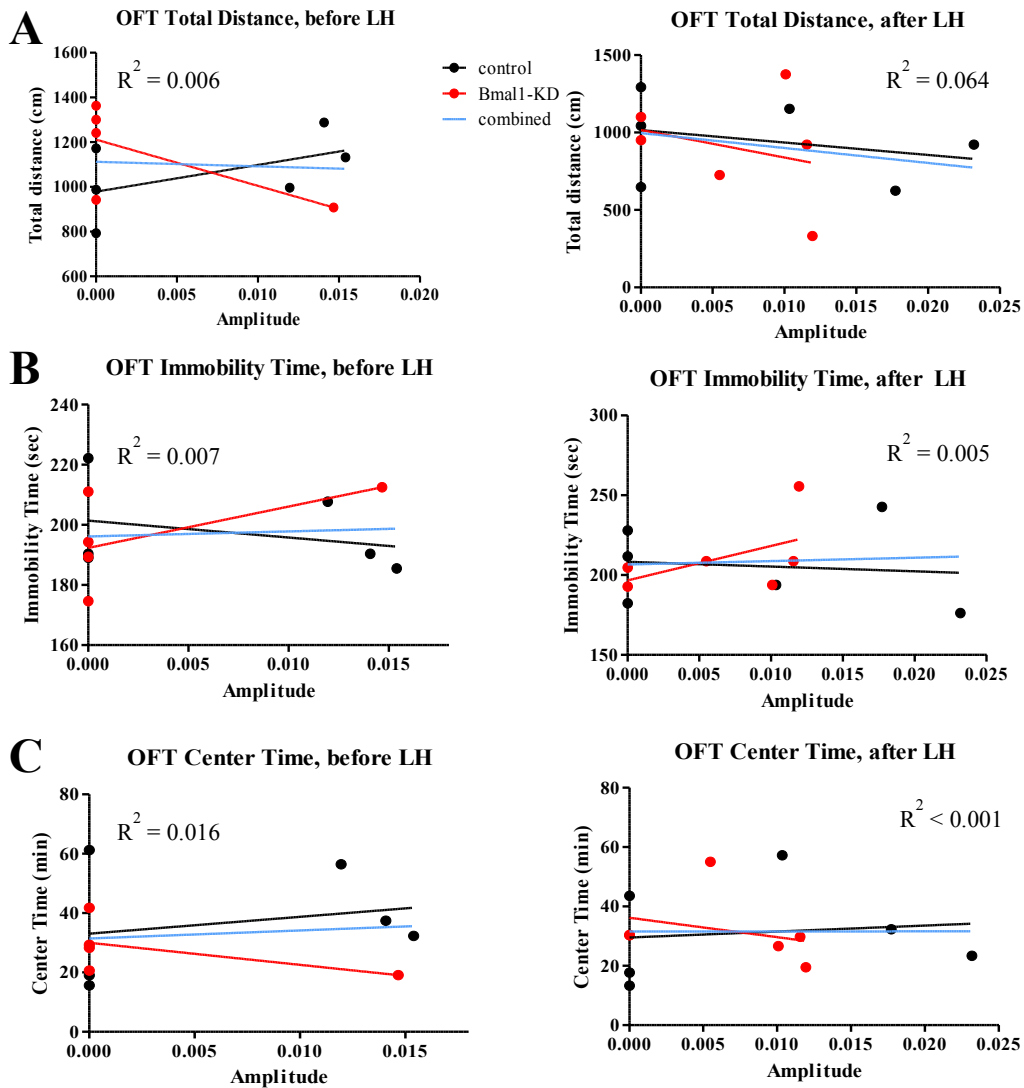


Figure 4: Open Field Test results from mice with BMAL1 knockdown in the NAc. For each graph, OFT data mouse is plotted with that mouse's PER2 rhythm amplitude of the PAG explant. The mice before ($n=11$) or after ($n=12$) LH are a different group of mice. *A*, the Total Distance each mouse moved in the OFT box over 5 minutes before or after LH. *B*, the total OFT immobility time of each mouse over 5 minutes before or after LH. *C*, the total time spent in the center of the box over 5 minutes before or after LH. Solid lines are linear regression lines; R^2 is of combined data.

Circadian Rhythm disruption in the PAG

Mice virally injected with AAV in the PAG were tested for sucrose preference, open field test, tail suspension test and learned helplessness to determine if there was a correlation between rhythmicity in the PAG and mood-related behavior. The AAV injections to knockdown BMAL1 did not impact the rhythmicity or rhythm amplitude of the PAG. This is likely due to virus leaving the PAG into nearby ventricles. However, the large natural variations in PAG rhythmicity are used to determine correlations of mood-related behavior and having robust or weak circadian rhythms in the PAG.

Learned Helplessness

The PAG rhythm amplitude did not significantly correlate to the LH escape latency (Fig. 5A, left). In addition, there was no correlation between the amplitude of a mouse's PAG rhythms and the number of failures to escape (Fig. 5A, right). Mice with lower PAG rhythm amplitudes did not develop more depression-like behavior in LH than mice with high rhythm amplitudes.

Tail Suspension

Mice with AAV injections in the PAG underwent TST either before only or before and after LH (Fig. 2A and 2C). TST immobility time and immobility latency before LH did not significantly correlate with the PAG rhythm amplitude (Fig. 5B, left). Similarly, when mice were subjected to TST after LH, BMAL1 knockdown in

the PAG or amplitude of the PAG did not correlate with TST immobility time or latency (Fig. 5B, right).

Sucrose Preference

For these results, mice were categorized based on low or high PAG rhythm amplitude (high was > 0.0144). Low rhythm amplitude in the PAG did not have a statistically significant effect on mice's sucrose preference compared to mice with a highly rhythmic PAG (Fig. 5C).

Open Field Test

Mice with AAV injections in the PAG underwent OFT for 5 minutes either before only, or before and after LH (Fig. 2A and 2C). OFT results before or after LH did not correlate to the rhythm amplitude of the PAG (Fig. 6). Mice with low and high amplitude showed similar results in total distance, immobility time and center time before and after LH (Fig. 6A, B, and C, respectively). Mice were also tested for center distance, rear time, and center latency, however these data were insignificant (data not shown).

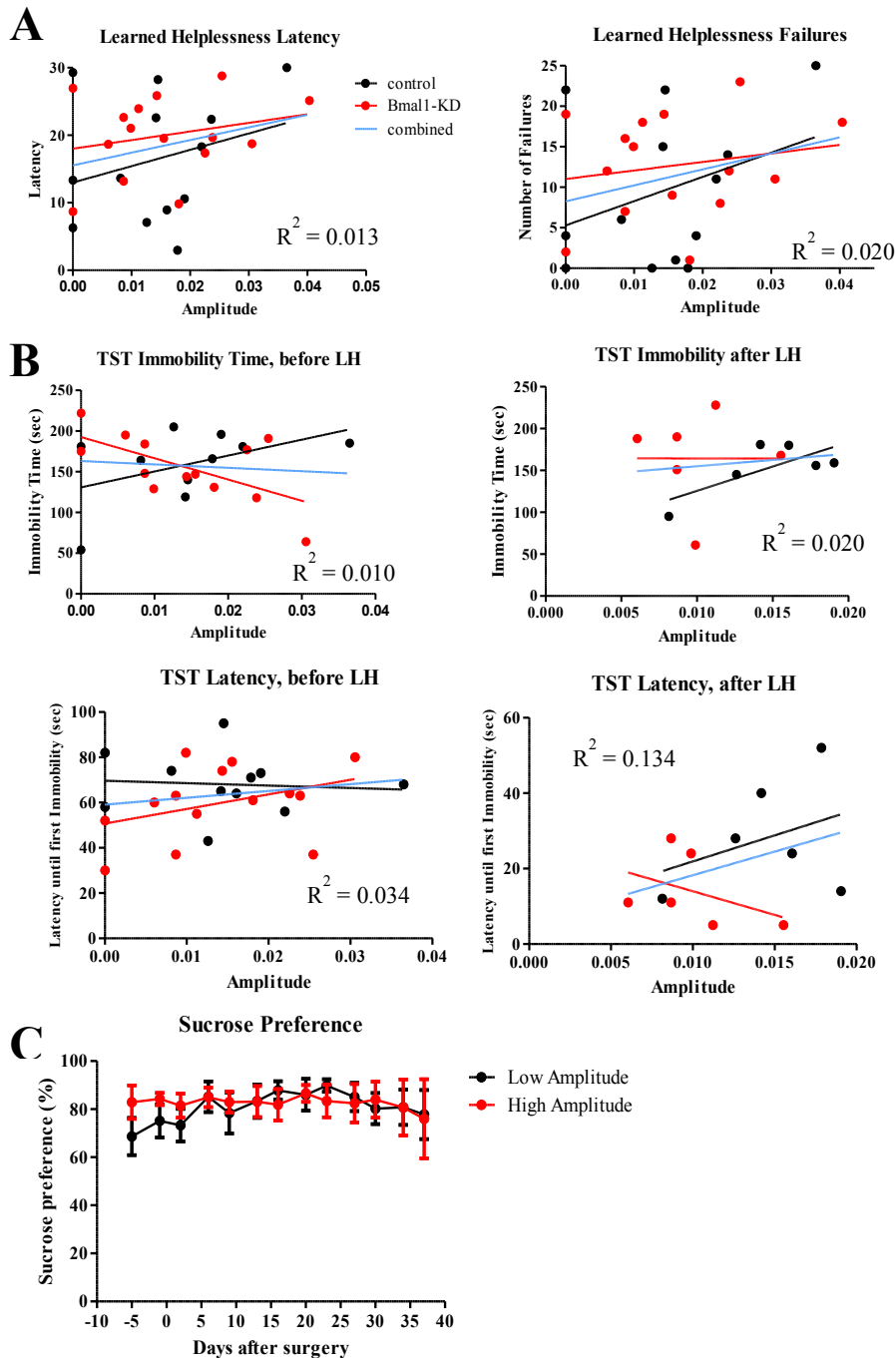


Figure 5: LH, TST and SP in mice with AAV injections in the PAG. A, LH escape latencies and number of escape failures correlated to PAG rhythm amplitude; solid lines are linear regression lines, R^2 is of combined data (n= 25). B, TST immobility time and latency before (n=23) and after (n=12) LH compared to PAG rhythm amplitude; solid lines are linear regression lines, R^2 is of combined data. C, Percentage of sucrose water consumed throughout experiment, grouped by low and high PAG rhythm amplitude (n=25); data analyzed two-way ANOVA with Bonferroni post-test.

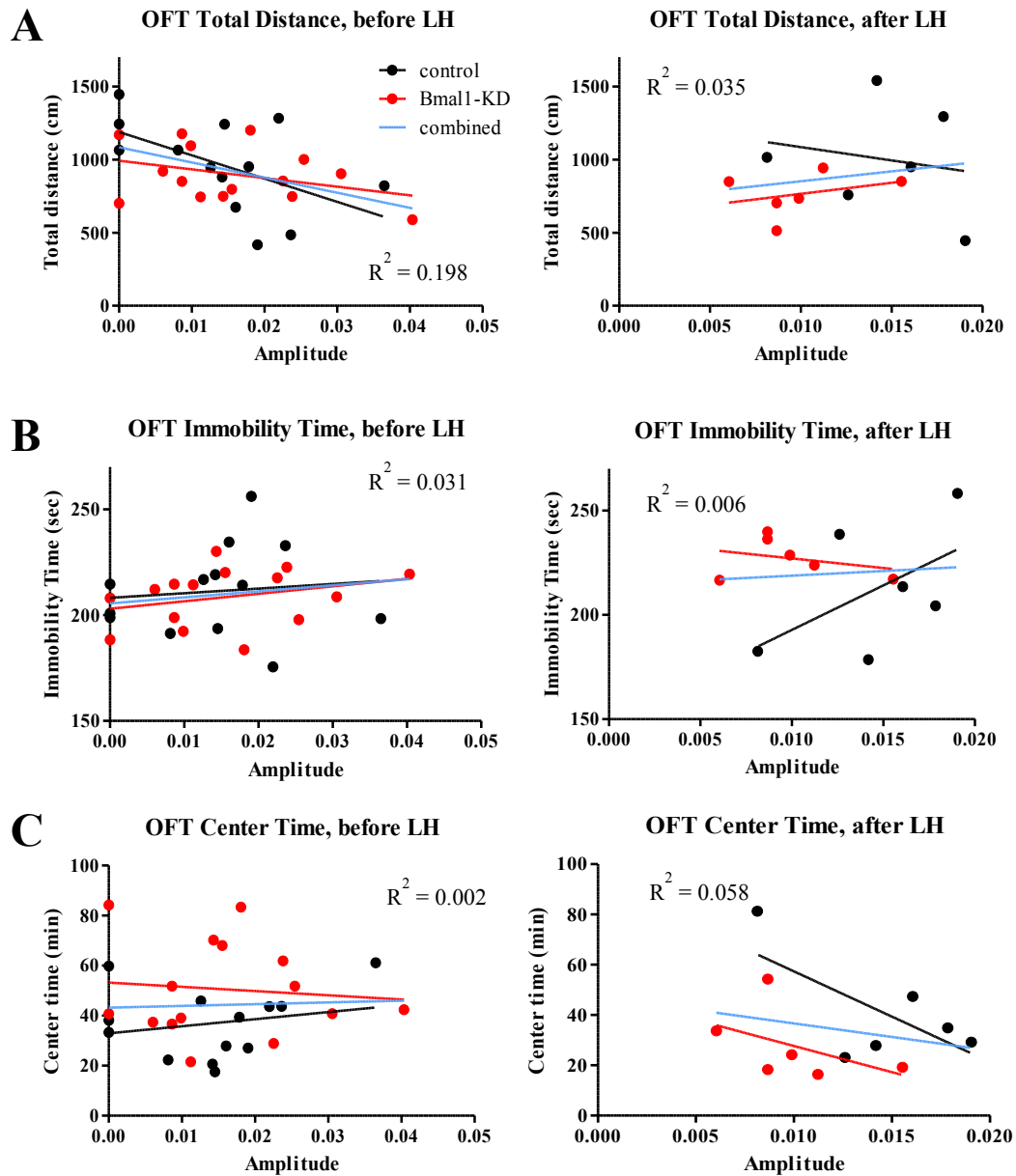


Figure 6: Open Field Test results from mice with BMAL1 knockdown in the PAG. For each graph, the OFT data for each mouse before ($n=23$) or after ($n=12$) LH is plotted with that mouse's Per2 rhythm amplitude in the cultured PAG slice. A, the Total Distance each mouse moved in the OFT box over 5 minutes before or after LH. B, the total Immobility Time of each mouse over 5 minutes before or after LH. C, the total time spent in the center of the box over 5 minutes, before or after LH. Solid lines are linear regression lines; R^2 is for combined data ($n=25$).

Circadian Rhythm disruption in the DR

Mice with AAV injections in the DR also underwent LH, OFT, TST and SP to determine if there was a correlation between rhythmicity in the DR and mood-related behavioral changes. There were fewer mice used for these injections, so it is more difficult to make confident correlations between behavior and amplitude. The AAV injections did not always impact the rhythmicity or rhythm amplitude of the DR, likely due to virus diffusing into ventricles. However, the large natural variation in DR rhythmicity is used to determine if there is a correlation between mood-related behavior and robust or weak circadian rhythms in the DR.

Learned Helplessness

There was no significant correlation between LH escape latency and the rhythm amplitude of the DR (Fig. 7A left). There was also no significant correlation between the amplitude of a mouse's DR rhythms and the number of failed escapes (Fig. 7A, right).

Tail Suspension

Rhythm amplitude in the DR did not significantly correlate to TST immobility time or immobility latency when TST was done before LH (Fig. 7B, left). In addition, when mice were subjected to TST after LH, rhythm amplitude of the DR did not correlate with TST immobility time or immobility latency (Fig. 7B. right). For mice that had an arrhythmic DR, there was a large variation in TST immobility time and latency after LH.

Sucrose Preference

For sucrose preference analysis, mice were grouped based on arrhythmic (amplitude = 0) or rhythmic (amplitude > 0) DR, independent of injection type. Mice that were injected with arrhythmic DR did not differ in the percentage of sucrose water consumed compared to mice with rhythms in the DR (Fig. 7C).

Open Field Test

Mice with AAV injections in the DR underwent OFT for 5 minutes either before or after LH (Fig. 8A, 8B). However, OFT results before and after LH did not correlate to the rhythm amplitude of the DR explant (Fig. 8). DR rhythm amplitude did not correlate with differences in total distance, immobility time and center time before and after LH (Fig. 8A, B, and C, respectively). While the R^2 values for the OFT results before LH are fairly high, it is difficult to interpret these graphs since there were so few mice involved in the study (Fig. 8). The p-values for these graphs were not significant. Mice were also tested for center distance, rear time, and center latency, however these data were also insignificant (data not shown).

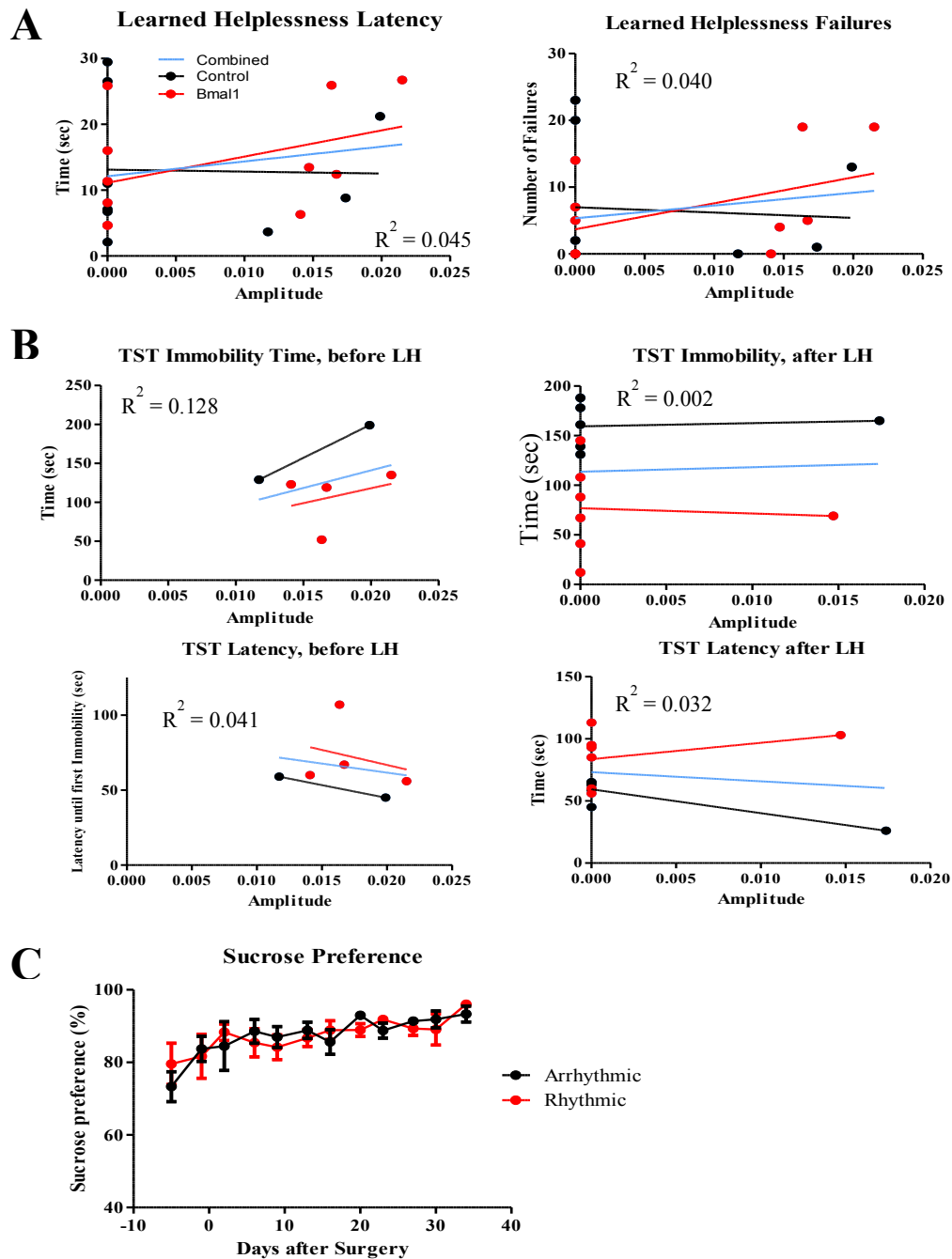


Figure 7: LH, TST, and SP results in mice with AAV injections in the DR. A, LH escape latency and number of escape failures correlated to DR rhythm amplitude ($n=20$). B, TST immobility time and immobility latency before ($n=6$) or after ($n=13$) LH, compared to DR rhythm amplitude. Solid lines are linear regression lines; R^2 is for combined data. C, Percentage of sucrose water consumed of total liquid consumed for mice with rhythmic or arrhythmic DR; data analyzed two-way ANOVA with Bonferroni post-test.

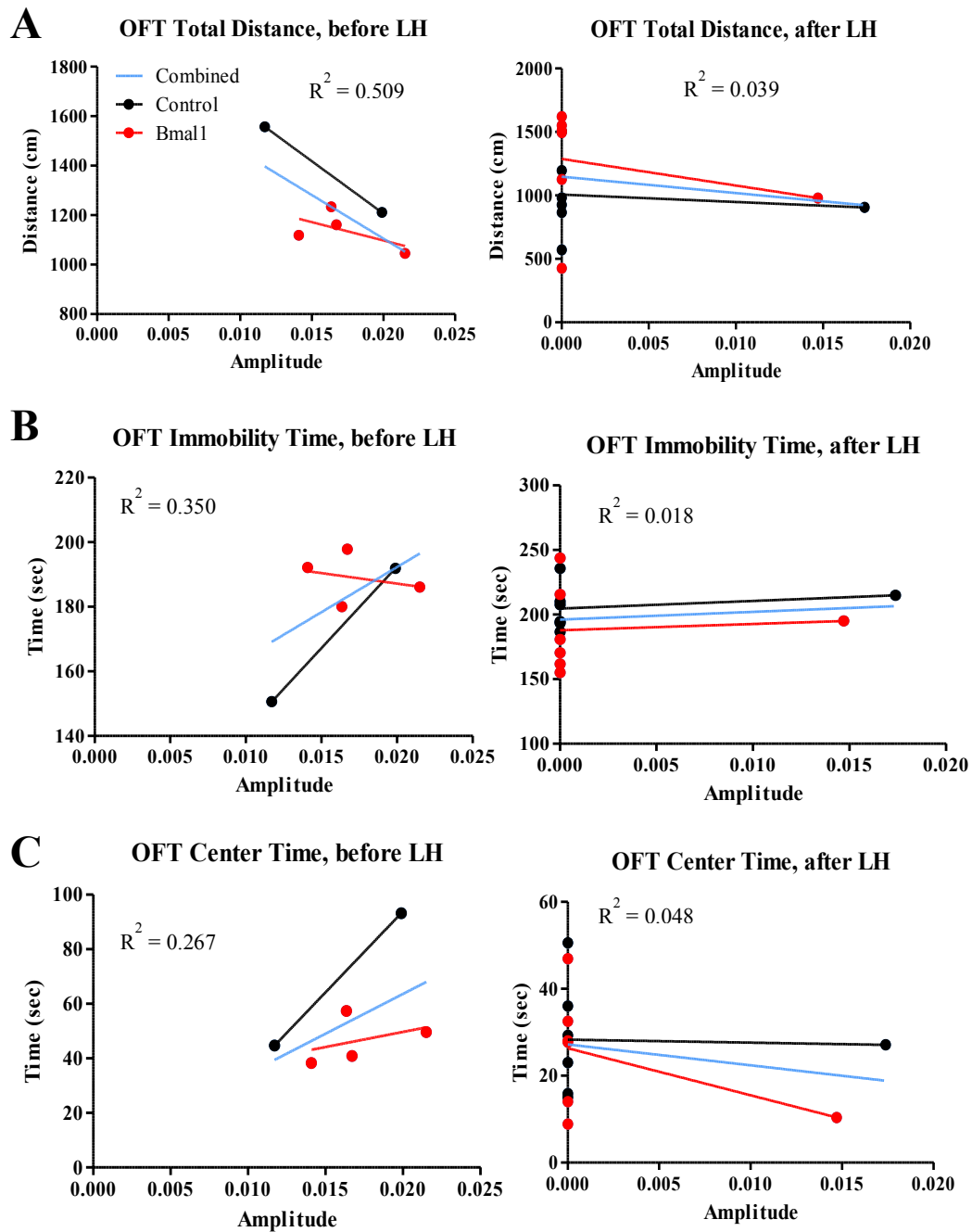


Figure 8: Open Field Test results from mice with BMAL1 knockdown in the DR. For each graph, the OFT data for before ($n=6$) or after ($n=13$) LH is plotted with the Per2 rhythm amplitude in the DR. *A*, the total distance each mouse moved in the OFT box over 5 minutes before or after LH. *B*, the total immobility time of each mouse over 5 minutes before or after LH. *C*, the total time spent in the center of the box over 5 minutes, before or after LH. Solid lines are linear regression lines; R^2 is for combined data.

Circadian Rhythm disruption in the SCN

The mice with AAV injections in the SCN were subjected to behavioral tests in the timeline described by Fig. 2C. AAV BMAL1-KD injections into the SCN were not always successful at eliminating rhythmicity, as seen by the spectrum of rhythm amplitudes for both BMAL1 and control mice (Fig. 10-12). However, BMAL1 mice had significantly lower PER2 rhythm amplitudes than control mice (Fig. 9). Disruption of circadian rhythms in the SCN causes a disruption of other peripheral clocks, most likely by desynchronizing these clocks from each other.

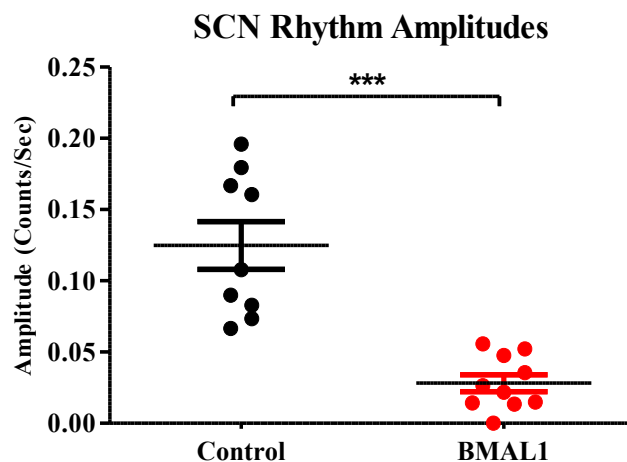


Figure 9: PER2 rhythm amplitudes from SCN explants of BMAL1-knockdown injected mice are significantly lower than controls. Rhythm amplitudes were determined using Lumicycle software. Data points are shown with average \pm SEM; $p^{***} \leq 0.001$, t-test.

Learned Helplessness

Circadian rhythm disruption in the SCN correlated with higher LH average escape latency and a higher number of escape failures in mice (Fig. 10). This is more easily seen when looking at the combined BMAL1 and control correlation line (shown

in blue), as the line shows a downward slope correlating high helplessness behavior with low rhythm amplitude (Fig. 10A). Plotted a different way, where BMAL1 and control mice were grouped together, LH escape latency and number of failures were significantly higher in BMAL1-injected mice (Fig 10B).

Tail Suspension

Disrupted rhythms in the SCN did not significantly correlate with immobility time or immobility latency in TST before LH (Fig. 11A, left). After LH, disturbed rhythms in the SCN also did not significantly correlate with TST immobility time or immobility latency (Fig. 11A, right). Since these mice underwent TST two times, it is unknown whether the second TST was affected by memory of doing TST before, by the stress of LH, or both. However, no significant differences were seen between TST results before or after LH.

Sucrose Preference

BMAL1 knockdown in the SCN did not significantly influence the mice's sucrose water preference throughout the entire experiment (Fig. 11B). After 3 weeks from the injection, when BMAL1 knockdown was in full effect, sucrose water consumption did not differ significantly between the BMAL1 and control groups (Fig. 11B).

Open Field Test

For the OFT done before LH, rhythm amplitude of the SCN did not correlate with total distance moved, immobility time, or center time results (Fig. 11A, B, C, left). After LH, OFT results changed only in the measurement of immobility time. For this measurement, lower rhythm amplitude correlated with lower immobility time in the OFT (Fig. 11B right). When comparing controls and BMAL1 knockdown mice, there was a significant decrease in immobility time during the second OFT for BMAL1 mice compared to controls. The other measurements of total distance and center time did not correlate with SCN rhythm amplitude after LH (Fig. 11A and C, right). Since these mice underwent OFT two times, it is not clear whether the difference seen in immobility time before and after LH are due to the inescapable stress of LH, the mouse remembering the OFT, or both.

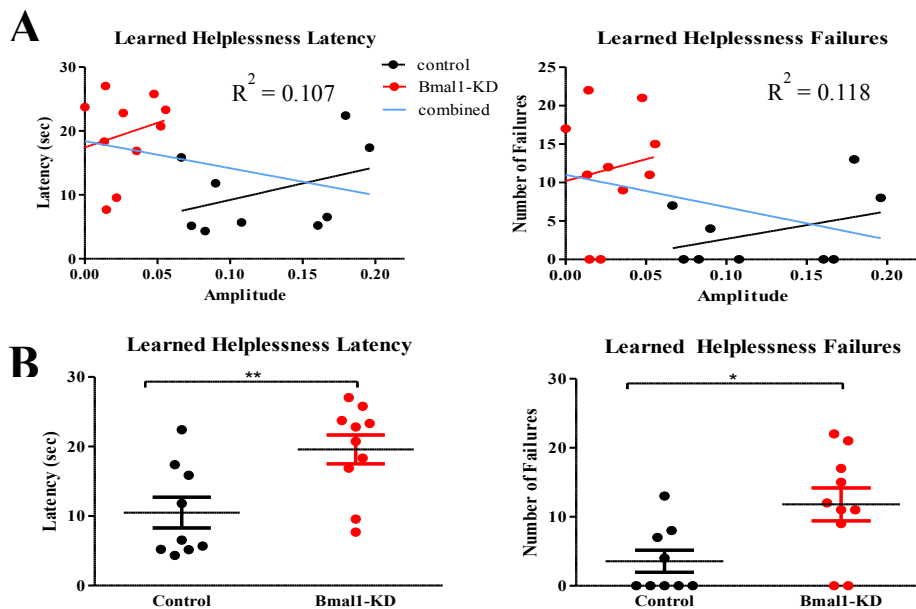


Figure 10: LH results from mice injected with AAV in the SCN. A, LH results of escape latency and number of escape failures were correlated with the SCN rhythm amplitude; solid lines are linear regression lines; R^2 is for combined data. B, BMAL1 and Control mice were compared for results in escape latency and number of escape failures; data are shown as average \pm SEM; ** $p \leq 0.01$, * $p \leq 0.05$, t-test.

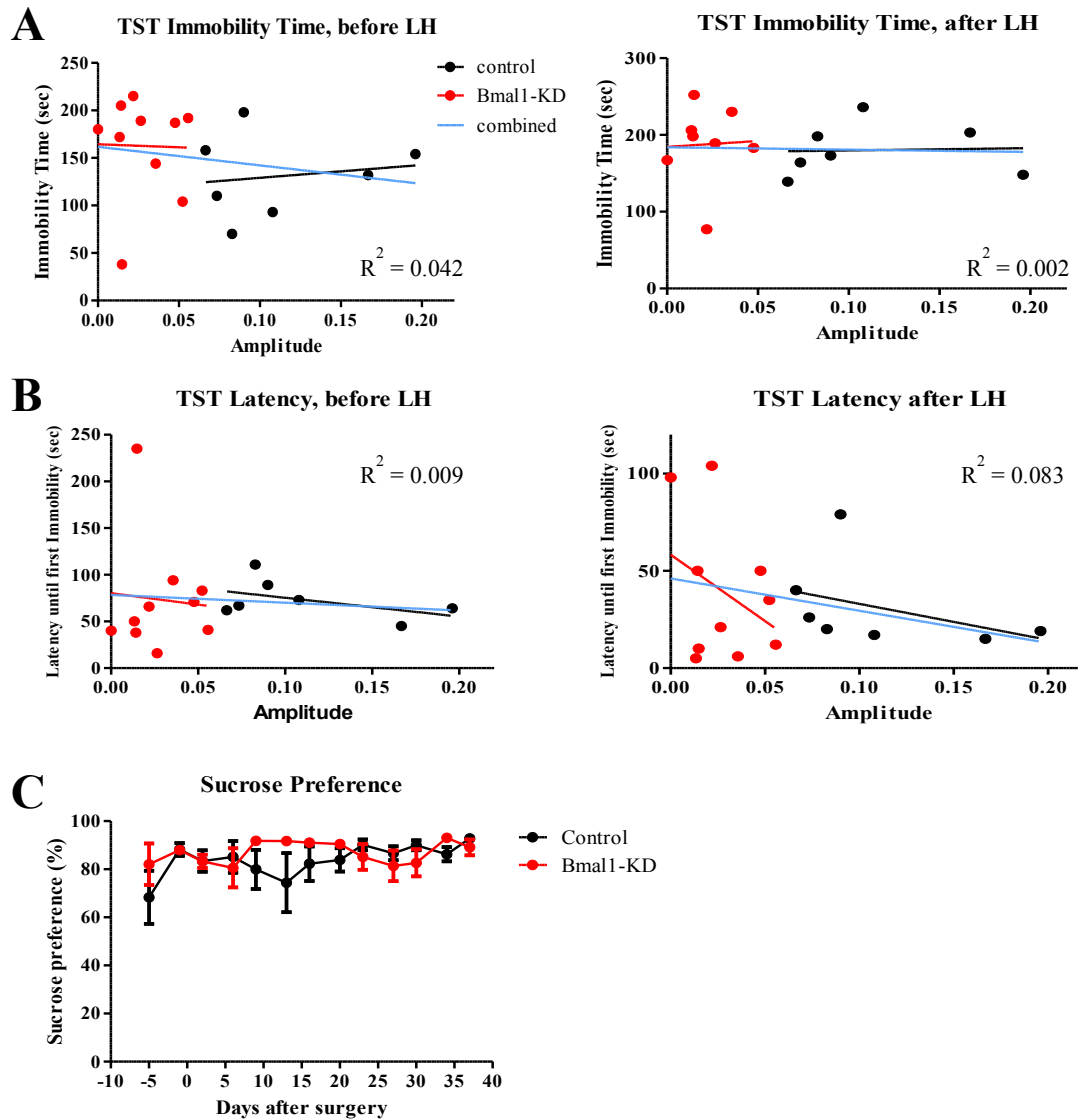


Figure 11: SCN BMAL1 knockdown mice performance in tail suspension and sucrose preference. A, TST results of immobility time and B, immobility latency compared to SCN rhythm amplitude; solid lines are linear regression lines; R^2 is for combined data. C, Percentage of sucrose water consumption over entirety of experiment of control-injected and BMAL-KD injected mice.

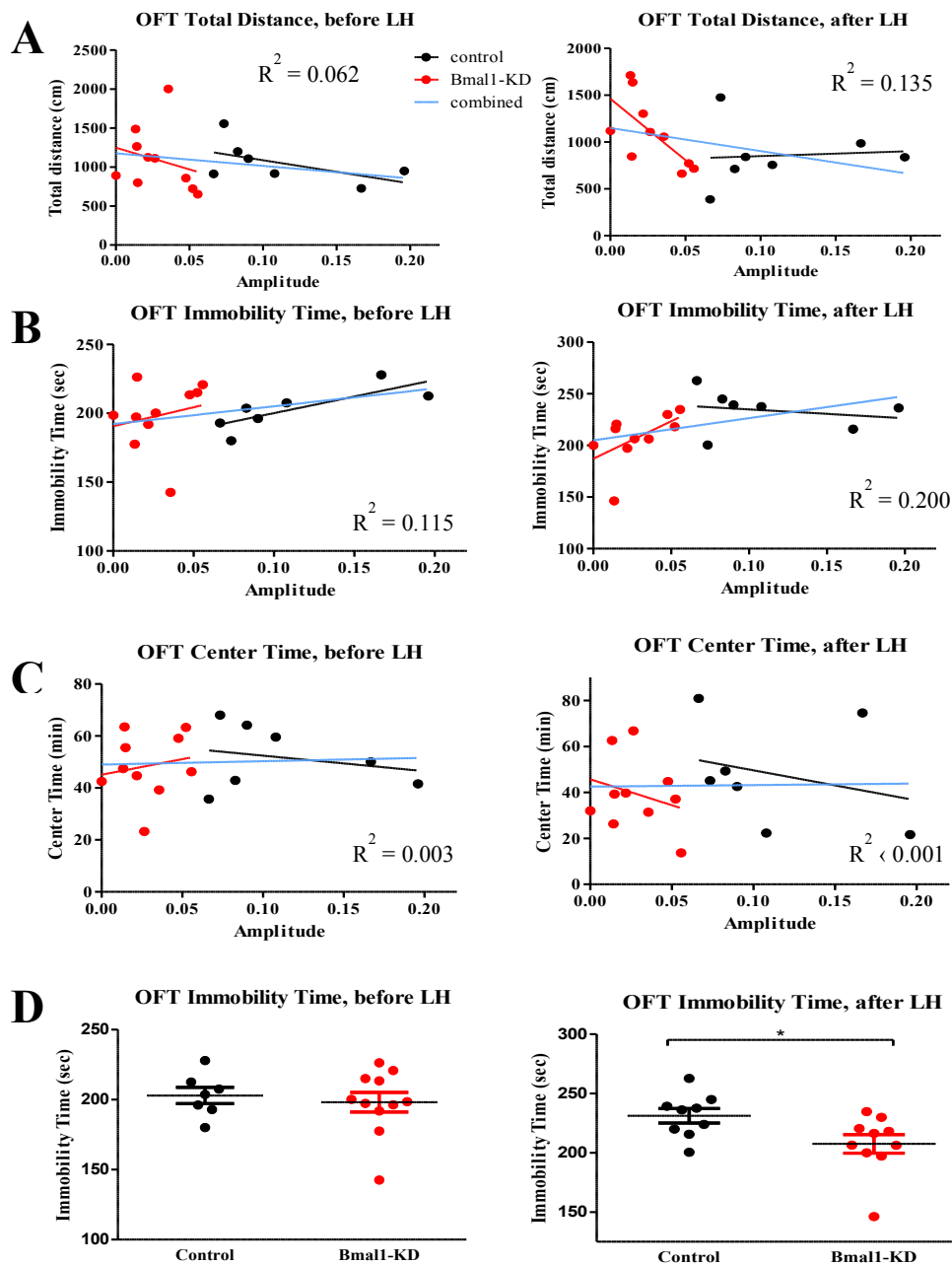


Figure 12: SCN BMAL1 knockdown Open Field Test results. Mice with AAV injections into SCN underwent OFT before and after LH. OFT results before and after LH are shown. A, the Total Distance mice moved during the first 5 min is plotted with the amplitude of the SCN slices' circadian rhythms. B, the immobility time over 5 min in OFT plotted against amplitude of the SCN slice. C, the amount of time spent in the center over 5 min plotted against amplitude of SCN slice. Solid lines are linear regression lines; R^2 is for combined data. D, the immobility time separated by BMAL1-knockdown and control groups. Data are shown as average \pm SEM; * $p \leq 0.05$.

5-HTR expression using qPCR

In addition to behavioral testing, mRNA was isolated from mice PAG slices to determine if there was a physiological difference in diurnal 5-HTR expression levels when circadian rhythms were lost. Due to procedural restrictions of culturing brain tissue slices, there was not enough 5HTR RNA in PAG slices to be detectable in qPCR. After 40 thermal cycles in the qPCR machine, no 5HTR RNA was detected. The housekeeping gene, B-actin did amplify according to the concentration of cDNA added to the well (data not shown).

Discussion

Circadian rhythm disturbance has been recognized in the symptoms of psychiatric mood disorder patients, but the mechanism of how circadian rhythms affects mood disorders remains unknown. Unpublished data in our has found a connection between a change in rhythmicity of the NAc, PAG, and DR with the development of helplessness in mice, but the directionality of this connection was not known. In the present study, one aim was to resolve the ambiguity between whether mood disorders are a consequence of disrupted rhythms or if they cause disrupted rhythms. Results from the present experiments show that mice with disrupted circadian rhythm synchronization in the entire brain, through BMAL1 knockdown in the SCN, develop depression-like behavior. However, if circadian rhythms of only one mood-related brain region are disturbed, or have lowered rhythm amplitude, there is no significant change in mood-related behavior. Another aim of this study was to determine if there was a physiological effect of having disrupted circadian rhythms by studying 5-HTR expression in arrhythmic and rhythmic PAG explants. Due to technical and methodological limitations, the 5-HTR RNA was not detectable in the qPCR experiment, so this question is still yet to be resolved.

SCN circadian disruption is sufficient for development of depression-like behavior

Mice behavior was examined after BMAL1 knockdown in the SCN. Since the SCN is the main circadian rhythm synchronizer in the mouse, knocking down rhythms in the SCN essentially disrupts rhythms in the entire brain. However, BMAL1 injection did not always lead to complete arrhythmicity of the SCN, likely because of

injection precision error or insufficient shRNA sequences. However, the knockout was sufficiently pronounced to significantly reduce the amplitude of PER2 rhythms in the SCN (Fig. 9). Interestingly, disrupted rhythms in the SCN from BMAL1 injection caused mice to develop greater depression-like behavior (Fig. 10). Mice that become helpless are more susceptible to uncontrollable stressors and develop mood-related behavioral changes. Mice that don't become helpless are more resilient against uncontrollable stressors. Therefore, disrupted circadian rhythms in the brain make mice more vulnerable to uncontrollable stressors and more likely to show depression-like behavior. In addition, a significantly reduced immobility time in OFT after LH was seen in mice with disrupted SCN rhythms (Fig. 12). Reduced immobility time could be associated with less freezing behavior or more exploratory behavior, but it is unclear whether this is indicative of depression-like behavior. However it is known that many patients with anxiety tend to be hyperactive, so this behavior could be associated with anxiety. Also, it is not certain whether this difference is due to the effects of inescapable stress of LH or due to memory, since OFT was done after LH. It is possible that mice with disturbed SCN rhythms remembered the OFT situation worse than mice without disturbed rhythms, since they spent more time exploring the environment.

SCN circadian disruption does not affect reward-seeking or despair-like behavior

Disruption of circadian rhythms in the SCN is not sufficient to alter reward-seeking behavior in mice, as tested by sucrose preference test (Fig. 11C). In addition, circadian rhythm disruption in the SCN is not sufficient to cause despair-like behavior

in mice, as tested by TST (Fig. 11A and B). This reveals that circadian oscillations in the brain are not important modulators of reward-seeking and despair-like behavior in mice, or at least these behaviors were not revealed by the tests.

Circadian disruption in the NAc, PAG or DR does not affect mood-related behavior.

Mice behavior was examined after creating localized circadian disruption in the NAc, PAG, and DR via BMAL1 knockdown. Not all BMAL1 knockdown injections led to arrhythmicity or reduced amplitude in these brain regions, likely due to AAV diffusion into ventricles. In addition, some control mice were arrhythmic or had lowered rhythm amplitude. This finding is normal based on results from previous studies that mice sometimes have naturally arrhythmic NAc, PAG or DR. However, since the manipulation to knockdown BMAL1 was not effective, no causal relationships could be made between BMAL1 knockdown and behavior. Mice that had disturbed rhythms in NAc, PAG, or DR did not display differences in depression-like behavior compared to mice with robust rhythms (Fig. 3A, 5A, and 7A). This shows that disrupted rhythmicity in these specific brain areas alone did not correlate with depression-like behavior. In addition, disturbed rhythm amplitudes in the NAc, PAG or DR was not sufficient to cause despair-like behavior in TST (Fig. 3B, 5B, and 7B). The sucrose preference test in mice with disturbed rhythms in the NAc, PAG or DR did not reveal changes in reward-seeking behavior compared to controls (Fig. 3C, 5C, and 7C). Lastly, disrupted rhythms in the NAc, PAG or DR was not sufficient for mice to develop anxiety-like behavior in the OFT (Fig. 4, 6, and 8). However, causality between circadian rhythms and behavior of mice cannot be determined since there

could be other variables involved that could be manipulating both rhythms and mood. A better method of inducing arrhythmicity in these brain areas is needed. Ideally, in future studies there would be a way to compare mood-related behavior between mice with completely arrhythmic and rhythmic NAc, PAG or DR via circadian rhythm manipulation. Another technical issue that occurred during these experiments is that it is often difficult to detect circadian rhythms in these brain areas. It could be possible that some of the brain areas were rhythmic, but the rhythmic expression was so low that it was not detectable.

5-HTR levels in PAG explants were not detectable by qPCR

5-HTR expression levels were examined to determine if there is a physiological effect of having disrupted circadian rhythms in the PAG, since serotonin is known to be involved in mood disorders. No 5-HTR expression was detected after amplification in qPCR, meaning not enough 5-HTR was in the samples initially for proper amplification. However, the housekeeping gene amplified normally, which shows that the RNA isolated and the cDNA made from the slices was intact and of good quality. Another more sensitive technique or a higher 5-HTR concentration is needed to determine the effects of rhythmicity and arrhythmicity on 5-HTR expression in the PAG or other brain areas.

Conclusion

Overall, findings from behavioral tests reveal that disturbing circadian rhythms in the entire brain, by reducing rhythm amplitude in the SCN via BMAL1 knockdown,

is sufficient to cause depression-like behavior in mice. However, disturbed circadian rhythms in the NAc, PAG, or DR were not correlated with the development of mood-related behavioral changes. One issue is that when SCN rhythms are disrupted, it is not known whether other brain areas are still rhythmic from external time cues, since some brain areas are also known to receive light information. In addition, the number of brain areas involved in mood-regulation and their clock function is not fully understood. To answer these questions, further studies could be done to disturb rhythms in different combinations of brain areas. More information would need to be known about how different areas of the brain are involved in psychiatric mood disorders and how these areas might interact to form a clock network. In addition, to determine physiological effects of having arrhythmic brain areas, a new method to determine neurotransmitter receptor expression could be tested. Because of restrictions within our lab and culturing tissues, new methods for determining serotonin receptor expression could not be accomplished. Overall, my research sets a foundation to further study the relationship between circadian rhythms and psychiatric mood disorders, and shows that disruption of whole-brain circadian rhythm synchrony can cause depression-like behavior in mice. This research study acts as a starting point in determining that circadian rhythm disturbance causes depression-like behavior, and will hopefully lead to developing psychiatric therapies focused on circadian rhythms in patients.

References

- Albrecht, U. (2012). Timing to perfection: The biology of central and peripheral circadian clocks. *Neuron*, *74*, 246–260.
- Bunger, M.K., Wilsbacher, L.D., Moran, S.M, Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, C.M., Takahashi, J.S., Bradfield, C.A. (2000). Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals. *Cell*. *103*, 1009-1017.
- Can, A., Dao, D. T., Terrillion, C. E., Piantadosi, S. C., Bhat, S., Gould, T. D. (2012). The Tail Suspension Test. *J. Vis. Exp.* *59*, e3769
- Chourbaji S, Zacher C, Sanchis-Segura C, Spanagel R, Gass P. (2005). Social and structural housing conditions influence the development of a depressive-like phenotype in the learned helplessness paradigm in male mice. *Behavioural brain research*; *164(1)*:100–6.
- Daan S, Pittendrigh CS. (1976) A Functional analysis of circadian pacemakers in nocturnal rodents. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*.*106(3)*:223–355.
- Dibner, C., Schibler, U., & Albrecht, U. (2010). The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. *Annual Review of Physiology*, *72*, 517–549.
- Graeff, F.G., Guimaraes, F.S., De Andrade, T.G.C.S., Deakin, F.W. (1996). Role of 5-HT in Stress, Anxiety, and Depression. *Pharmacology Biochemistry and Behavior*. *44(1)*, 129-141.
- Güler, A.D., Ecker, J.L., Lall, G.S., Haq, S., Altimus, C.M., Liao, H-W., Barnard, A.R., Cahill, H., Badea, T.C., Zhao, H., Hankins, M.W., Berson, D.M., Lucas, R.J., Yau, K-W., Hattar, S. (2008). Nature. Melanopsin cells are the principle conduits for rod-cone input to non-image-forming vision. *453*, 102-105.
- Landgraf, D., McCarthy M.J., and Welsh D.K. (2014). The Role of the Circadian Clock in Animal Models of Mood Disorders. *Behavioral Neuroscience*. *128(3)*, 344-59.
- Landgraf D., Long J., Der-Avakian A., Streets M., Welsh D.K. (2015) Dissociation of Learned Helplessness and Fear Conditioning in Mice: A Mouse Model of Depression. *PLoS ONE* *10(4)*: e0125892.
- Li, J.Z., Bunney, B.G., Meng, F., Hagenauer, M.H., Walsh, D.M., Vawter, M.P., Evans, S.J., Choudary, P.V., Cartagena, P., Barchas, J.D., Schatzberg, A.F., Jones,

E.G., Myers, R.M., Watson, S.J., Akil, H., Bunney, W.E. (2013). Circadian Patterns of Gene Expression in the Human Brain and Disruption in Major Depressive Disorder. *Proceedings of the National Academy of Sciences*. *110.24*, 9950-95.

McCarthy, M.J., and Welsh, D.K. (2012). Cellular Circadian Clocks in Mood Disorders. *Journal of Biological Rhythms* *27.5*, 339-52.

Partch, C.L., Green, C.B., Takahashi, J.S. (2014). Molecular architecture of the mammalian circadian clock. *Cell Biology*. *24,2*. 90-99.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. *29(9)*, 2002-2007.

Schmidt, T.M., Chen, S., Hattar, S. (2011) Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. *Cell: Trends in Neurosciences*. *34(11)*, 572-580.

Silver, R., Kriegsfeld, L.J. (2014). Circadian rhythms have broad implications for understanding brain and behavior. *European Journal of Neuroscience*. *39(11)*, 1866-1880.

Spencer, S., Falcon, E., Kumar, J., Krishnan, V., Mukherjee, S., Birnbaum, S.G., McClung, C.A. (2013). Circadian genes *Period 1* and *Period 2* in the nucleus accumbens regulate anxiety-related behavior. *The European Journal of Neuroscience*. *32(2)*, 242-50.

Welsh, D.K., Takahashi, J.S., Kay, S.A. (2010). Suprachiasmatic Nucleus: Cell Autonomy and Network Properties. *Annual Review of Physiology*. *71*, 551-577.

Wulff, K., Gatti, S., Wettstein, J.G., Foster, R.G. (2010). Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nature Reviews*. *11*, 589-599.

Ye, R., Selby, C.P., Chiou, I.O., Gaddameedhi, S., Sancar, A. (2014). Dual modes of CLOCK:BMAL1 inhibition mediated by Cryptochrome and Period proteins in the mammalian circadian clock. *Genes and Development*. *28*, 1989-1998.

Yoo S.H., Yamazaki S., Lowrey P.L., Shimomura K., Ko C.H., Buhr E.D. (2004) PERIOD2::LUCIFERASE realtime reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences of the United States of America*. *101(15)*:5339-46.

Welsh D.K., Yoo S.H., Liu A.C., Takahashi J.S., Kay S.A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Current biology*: *CB.14(24)*. 2289-95.