Environmentally-Induced Circadian Disruption and Alcohol Consumption: Shared and Distinct Effects in Female Rats

Christiane Meyer

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By: Christiane Meyer

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Signed by the final examining committee:

_____ Chair
Dr. Philippe Caignon

_____ External Examiner
Dr. Dietmar Weinert

_____ Internal Examiner
Dr. Richard Courtemanche

_____ Thesis Co-supervisor
Dr. Dajana Vuckovic

_____ Thesis Co-supervisor
Dr. Wayne Brake

_____ Principal Thesis Supervisor
Dr. Shimon Amir

Approved by _____
Dr. Felice Yuen, Graduate Program Director

July 25, 2023 _____
Dr. Pascale Sicotte, Dean of the Faculty of Arts and Science

Abstract For PhD

Environmentally-Induced Circadian Disruption and Alcohol Consumption: Shared and Distinct Effects in Female Rats

Christiane Meyer, PhD

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The disruption of daily rhythms in physiology is a widespread concern in modern society and affects individuals across the entire population. Repeated rhythm disruption has detrimental effects on health and is known to be a risk factor for the development of mental conditions, including alcohol abuse and mood disorders. This results in an intricate clinical picture, as affective disorders are often comorbid with pathological alcohol consumption. Furthermore, affective behavior may also induce alcohol consumption or relapse, and both factors can affect daily rhythms in physiology, which may exacerbate the conditions ultimately. While there is accumulating evidence on the negative consequences of circadian disruption in males, knowledge in females is limited. This project aims to bridge this gap by investigating the association between circadian desynchronization and alcohol consumption, focusing on behavior and physiology in female rats.

To model environmentally-induced circadian disruption, we exposed female rats to either a short-day light cycle or repeated shifts of the light/dark cycle and investigated alcohol drinking behavior, mood-related behavioral changes, and physiology to study the effect of chronodisruption. Moreover, we analyzed changes in gene expression in brain regions associated with the reward system following circadian disruption and alcohol consumption to understand the underlying mechanism of displayed behaviors.

The results demonstrate that exposure to aberrant light conditions disrupts the circadian system and impairs physiological processes such as the estrous cycle. Strikingly, the sole effects of the light conditions on mood-related behavior and alcohol consumption were minor, despite changes in gene expression in the brain regions related to reward processing. However, chronodisrupted females displayed changes in mood-related behavior under alcohol abstinence, indicating that the

combined impact of circadian desynchrony and stressors like abstinence are risk factors in the development of mental conditions, that may affect drinking behavior or relapse subsequently.

The outcomes of this thesis expand our understanding of the effects of chronodisruption in females and establish a foundation to further explore the complex relationship between circadian disruption, alcohol consumption, and their effects on mental health. This knowledge can guide future studies and interventions targeting mood disorders and alcohol misuse in females, especially in the context of shiftwork.

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Contribution of Authors

Chapter 1 includes a published manuscript, that is co-authored by Konrad Schoettner and Shimon Amir. CM and SA conceived and designed the experiments. CM conducted the experiments and was assisted by KS. CM analyzed and interpreted the data with input from KS and SA. CM did the data visualization. CM wrote the manuscript and revised it by SA and KS. SA supervised the project. All authors contributed to the article and approved the submitted version.

Chapter 3 is a draft manuscript that is yet to be submitted to an academic journal (in preparation). This manuscript is co-authored with Konrad Schoettner and Shimon Amir. CM and SA conceived and designed the experiments. CM conducted the experiments and was assisted by KS. CM analyzed and interpreted the data with input from KS and SA. CM did the data visualization. CM wrote the manuscript and revised it by SA and KS. SA supervised the project. All authors contributed to the article and approved the submitted version.

All authors reviewed the final manuscripts and approved the contents.

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List of Abbreviation

ANOVA	Analysis of variance
AUD	Alcohol use disorder
Bmal1	Aryl hydrocarbon receptor nuclear translocator-like gene
BAML1	Bmal1 protein
Clock	Circadian locomotor output cycles kaput gene
CLOCK	Clock protein
Cry1	Cryptochrome 1 gene
CRY1	Cryptochrome 1 protein
Cry2	Cryptochrome 2 gene
CRY2	Cryptochrome 1 protein
CTRL	Control group
DS	Dorsal striatum
EPM	Elevated plus-maze
Erβ	Estrogen receptor β gene
GluN2B	Glutamate ionotropic receptor NMDA type subunit 2B gene
HAD	High-alcohol-drinking
HPA	Hypothalamic-pituitary-adrenal
IAE20%	Intermittent alcohol exposure paradigm
IS	Interdaily stability
IV	Intradaily variability
Kcnn2	Small conductance calcium-activated potassium channel protein 2 gene
LD	Light-dark
LD22	11:11 hour light-dark cycle
MBT	Marble burying task
mGluR5	Metabotropic glutamate receptor subtype-5 gene
MSN	Medium spiny neurons
NAc	Nucleus accumbens

NMDA	N-methyl-D-aspartate receptor
OFT	Open field test
Per2	Period homolog 2 gene
PER2	Period homolog 2 protein
PFC	Prefrontal cortex
PR	Progesterone receptor gene
RA	Relative amplitude
Rev-erbα	Nr1d1: nuclear receptor subfamily 1, group D, member 1 gene
REVERBα	Rev-erbα protein
SCN	Suprachiasmatic nucleus
SEM	Standard error of the mean
SHIFT	Chronic advanced phase shift paradigm
SPT	Sucrose preference test
TNFα	Tumor necrosis factor-alpha gene
ZT	Zeitgeber time

Introduction

Alcohol misuse: A widespread public health concern

Alcohol abuse is a widespread public health concern with social and economic ramifications. Alcohol use disorder (AUD) affects over 280 million people worldwide (Glantz et al., 2020), including more than 18% of the Canadian population (Statistics Canada, 2013). Harmful consumption of alcohol increases the risk of suffering from numerous adverse consequences, including injury, disease, and premature death (Carvalho et al., 2019). The recent increase in the prevalence of women with AUD has unique serious adverse health consequences, given that women experience a more rapid progression of their addiction than men (Greenfield et al., 2010). Although the causes of alcohol abuse are multifactorial, including genetic, socio-economical, and psychological factors, disruption of daily rhythms such as the sleep-wake cycle is undeniably a contributing factor to the development of alcohol-abusive behaviors (Barko et al., 2019; Kendler et al., 2016).

In recent years, accumulating evidence suggests a bidirectional relationship between rhythm disruption and excessive alcohol consumption. Individuals who experience frequent desynchronization of their day-to-day activities with their rhythmic environment, like people working in rotating shifts, are susceptible to developing neuropsychiatric disorders including substance abuse and addiction (Logan & McClung, 2018; Richter et al., 2021). In turn, excessive drinking disrupts rhythmic processes such as the synthesis and release of hormones at specific times of the day, or the regulation of daily changes in body temperature and sleep (McCulley et al., 2013; Trujillo et al., 2011), which ultimately increases the risk of developing AUD and alcohol relapses during withdrawal. Thus, daily or circadian rhythms are considered important determinants in alcohol abuse and addiction.

The importance of circadian rhythms

Daily changes in the geophysical environment have imposed a unique selection pressure on living organisms over millions of years. Predictable alterations of environmental conditions such as the solar light/dark cycle create temporal niches for distinct biological processes which repeat in a circa 24h interval. These so-called circadian rhythms, which are generated by a highly conserved molecular mechanism (circadian clock), enable organisms to anticipate upcoming environmental changes rather than simply reacting to them (Paranjpe & Sharma, 2005). This

ensures that distinct biochemical, physiological, and behavioral processes are executed at a biologically relevant time of the day. Moreover, the temporal segregation of biological functions and organismal activities within the organism or even within certain organs and tissues is highly adaptive as it regulates metabolic demands selectively and efficiently and thus confers to systemic homeostasis and the overall fitness of the individual (Sharma, 2009; Vaze & Sharma, 2013). Likewise, a vast body of evidence shows that disruptions of circadian rhythms are associated with a higher risk of suffering from various disorders and diseases (Neves et al., 2022; Sato & Sato, 2023; Stowe & McClung, 2023).

At the molecular level, circadian clocks generate rhythms through highly conserved autoregulatory transcriptional-translational feedback loops (TTFL), which comprise a set of circadian clock genes and their protein products (Takahashi, 2016). At the core, heterodimers of BMAL1 (brain and muscle ARNT-like 1) and CLOCK (circadian locomotor output cycles kaput) activate the transcription of *Per* (period) and *Cry* (cryptochrome) genes, whose protein products repress their own transcription through inhibition of the BMAL1/CLOCK transcriptional activity. The core loop repeats every 24 hours and is stabilized through auxiliary loops in the circadian clockwork. Besides driving 24-h oscillations of core clock gene expression, other targets, so-called clock-controlled genes (CCGs) are rhythmically expressed and provide the required output of the circadian clock (Bozek et al., 2009). Up to 43% of all protein-encoding genes are influenced by the circadian clock (Zhang et al., 2014), which progressively manifests from cellular rhythms and rhythms of physiological processes to complex endpoints like behavior.

Although circadian clocks are virtually present in each cell of the organism, they form a hierarchically organized network called the circadian system. In mammals, the central circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Reuss, 1996). Because the intrinsic period of this cell-autonomous oscillator deviates from the exact 24-h environment, it has to be corrected or synchronized by external cues to provide stable and high-amplitude rhythmic signals for extra-SCN oscillators in the brain and peripheral clocks distributed throughout the organism. The light/dark cycle is the most prominent external cue, the so-called zeitgeber, to which the SCN entrains, which is received through retinal projections and integrated into the molecular circadian clockwork (Rollag et al., 2003). Rhythmic SCN output is

distributed by neuronal and humoral factors throughout the organism to provide timing cues to peripheral systems, which is crucial for the timing and temporal segregation of various body functions.

It is evident that the level of temporal alignment of circadian rhythms for both, the external environment (external alignment) and biochemical and physiological processes within the organism (internal alignment) are critical determinants of health and disease (Vetter, 2018). Repeated desynchrony or misalignment of circadian rhythms has been linked to the etiology of various neurological and neuropsychiatric disorders, including AUD (Arendt & Marks, 1982; Logan & McClung, 2018).

The link between circadian disruption and alcohol consumption

Circadian disruption, either through exposure to aberrant light/dark conditions or through perturbations of the molecular circadian clockwork, desynchronizes the biological timing of biochemical, physiological, and behavioral functions, which has been associated with altered alcohol consumption in humans and animal models.

Late chronotypes (i.e., individuals whose mid-sleep time is delayed compared to the average population) are known to bear a higher risk for substance abuse (Gulick & Gamsby, 2018). This is likely because they experience circadian misalignment through imposed social obligations (e.g., work schedules) creating a mismatch between biological preferred activity and resting times and their environment. Moreover, clinical studies have shown that shift work conditions, such as rotating schedules and nighttime employment are linked to binge drinking disorder. (reviewed by Richter et al., 2021).

Studies conducted on animals have confirmed the connection between abnormal light/dark conditions and alcohol consumption in rodents. This association varies depending on sex, light schedule, and alcohol-drinking paradigm (Clark et al., 2007; Rosenwasser et al., 2010, 2013). However, further research is needed to explore the underlying causes, such as changes in the expression of circadian clock genes in peripheral systems, which may contribute to abnormal alcohol consumption. Human studies have also indicated the possibility of altered circadian gene expression in individuals with AUD (Huang et al., 2010). However, it remains unclear whether this is a cause or a consequence of problematic alcohol consumption.

Genome-wide association studies in humans determined genetic variants of the circadian genes *Bmal1*, *Per1,* and *Per2* which are linked to altered alcohol drinking behavior (Comasco et al., 2010; Dong et al., 2011; Kovanen et al., 2010). In mice, global knockout of the circadian clock genes *Per2* or *Clock* promoted alcohol consumption in association with altered reward responses (Ozburn et al., 2013; Spanagel, Pendyala, et al., 2005), whereas *Cry1/2* double-knockout reduced the preference for alcohol (Hühne et al., 2022).

In addition to the influence of environmental and molecular factors on the circadian system as direct determinants for abnormal alcohol consumption, indirect factors may also contribute to changes in drinking behavior. Numerous neurobiological and physiological systems that influence affective behaviors have overlapping features with the reward circuit, including the critical role of circadian clocks and clock genes in their function. Circadian rhythm disruption is linked to the emergence of anxiety and depression-like symptoms (Bedrosian & Nelson, 2017). Mood disorders are common comorbidities in AUD patients, where episodes of mood dysregulation may accelerate increased alcohol intake behavior and trigger alcohol relapse (Yang et al., 2018). Additionally, alterations in the molecular pathways associated with the circadian system such as glutamatergic and sex hormone signaling, as well as inflammatory processes, have been linked to altered alcohol consumption in rodents (Eisenhardt et al., 2015; Feldman et al., 2020; Ho et al., 2019). Hence, circadian disruption may have an impact on physiological and behavioral systems that ultimately lead to altered alcohol-drinking behavior.

Sex differences – What about females?

Sex differences in alcohol consumption have been described in both human and animal models. Although previous work suggests that men consume more alcohol than women, this view has been challenged over the past years (Vatsalya et al., 2016). Research has found that women are more likely to develop alcohol-related issues at lower drinking levels and earlier than men. Alcohol mainly stays in body water, and since women have less amount of water in their bodies, drinking the same amount of alcohol as a man of the same weight can result in a higher blood alcohol concentration for women, which puts them at a greater risk of alcohol-related consequences. Studies have shown that women are more likely to experience hangovers and alcohol-induced blackouts than men (Vatsalya et al., 2018). Moreover, the negative effects of abusive alcohol consumption differ tremendously between sexes (Erol & Karpyak, 2015).

The role of the circadian system in relation to sex-specific differences in alcohol consumption in humans is not fully understood. While there is agreement that shiftwork can contribute to problematic alcohol consumption, the impact on females is less clear due to limited research that often excludes consideration of sex as a factor (Barlek et al., 2022; Mamlouk et al., 2020). Studies focused solely on female shift workers are scarce and yield conflicting results (Richter et al., 2021). This sex bias in clinical and animal research is problematic, as male and female neurobiology and physiology are distinct and influential beyond sex-specific behaviors (McCarthy et al., 2012). Neglecting to account for sex as an experimental variable, whether in a single study or across a research program, may impede important scientific discoveries and potentially harm the health outcomes of all genders.

Research on pre-clinical models has shown that the circadian timing system has sex-specific molecular variations that affect the behavior and physiology of both males and females (Yan & Silver, 2016). These differences may contribute to the higher alcohol consumption and preference for alcohol in female rodents compared to males (Rizk et al., 2022). The expression of circadian clock genes in reward-related brain regions plays a critical role in mediating these sex differences in alcohol consumption (de Zavalia et al., 2022). However, it is unclear how disruptions to circadian rhythms affect alcohol consumption patterns in females. Studies suggest that females may be more vulnerable to drug use and the development of excessive drug-taking behavior (Becker & Koob, 2016), and disruption of circadian rhythms is a risk factor for the development of AUD in females, particularly through dysregulation of rhythms in reward-associated brain regions.

Most previous research has primarily focused on male subjects (Eliot et al., 2023; Zucker & Beery, 2010), leaving implications regarding the effects of circadian disruption on alcohol consumption, associated mood-related behavior, and molecular processes in females poorly understood. Female animals were typically neglected because their reproductive cycle and corresponding hormonal state may affect the outcomes of certain variables, such as mood-related measures, motivation, or drug intake. As a result, medical advice and treatment options for females have been primarily based on data collected from pre-clinical studies conducted on males. However, more and more research suggests that it is critical to study sex independently from each other to gain a better understanding of gender differences in disease etiology, which is

important to develop targeted therapy measures to mitigate the deleterious effects of chronodisruption in females.

Hypothesis

This research project explores the intricate relationship between environmentally-induced circadian disruption and alcohol consumption, with a specific focus on their effects on female behavior and physiology. Our investigation is structured around three core hypotheses, each building upon the other to form a cohesive research narrative.

First and foremost, we hypothesize that the combined influence of environmentally-induced circadian disruption and alcohol consumption will exert a discernible influence on emotional behavior and physiological responses in female rats. Our second hypothesis advances the notion that the implementation of circadian-disruptive Light-Dark Schedules will precipitate changes in alcohol intake behavior. Importantly, these alterations are expected to be contingent upon the degree of internal desynchronization within the circadian system. Lastly, we anticipate the identification of molecular alterations stemming from long-term internal circadian disruption, which aligns with the observed behavioral and physiological outcomes.

Chapter 1 explores the effects of experimental LD conditions and alcohol consumption on female rats. It examines fluid intake behavior, emotional and reward-related behaviors during alcohol abstinence, and physiological parameters such as body weight and estrus robustness. This chapter contributes to our understanding of the impact of chronodisruption on alcohol intake and mood-related behaviors during alcohol abstinence in female rats.

Chapter 2 focuses on evaluating the relationship between chronodisruption, alcohol consumption, and their correlation with behavioral outcomes. By elucidating these relationships, this analysis contributes to our understanding of how internal desynchronization may influence an individual's drinking behaviors and subsequent behavioral responses.

Finally, **Chapter 3**, examines the molecular responses of internal desynchronization and alcohol intake on clock genes and cell-signaling genes in brain areas involved in the reward system. This chapter sheds light on the underlying molecular mechanisms that mediate the effects of circadian disruption on alcohol intake behavior in female rats.

In summary, this thesis represents an important first step in understanding the effects of circadian disruption on alcohol intake behavior, and how this influences myriad behavioral and physiological processes in females. The study contributes to the existing literature on the topic and holds implications for the development of interventions to prevent alcohol abuse in females.

Chapter 1: The effects of circadian desynchronization on alcohol consumption and affective behavior during alcohol abstinence in female rats

Christiane Meyer, Konrad Schoettner, Shimon Amir

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Author Contributions

CM and SA conceived and designed the experiments. CM conducted the experiments and was assisted by KS. CM analyzed and interpreted the data with input from KS and SA. CM did the data visualization. CM wrote the manuscript and revised by SA and KS. SA supervised the project. All authors contributed to the article and approved the submitted version.

1.1 Abstract

Disruption of circadian rhythmicity distorts physiological and psychological processes and has major consequences on health and well-being. A chronic misalignment within the internal time-keeping system modulates alcohol consumption and contributes to stress-related psychiatric disorders which are known to trigger alcohol misuse and relapse. While there is growing evidence of the deleterious impact of circadian disruption on male physiology and behavior, knowledge about the effect in females remains limited. The present study aims to fill the gap by assessing the relationship between internal desynchronization and alcohol intake behavior in female rats.

Female Wistar rats kept under standard 24-h, 22-h light-dark conditions, or chronic 6-h advanced phase shifts, were given intermittent access to 20% alcohol followed by an extended alcohol deprivation period. Alcohol consumption under altered light-dark (LD) conditions was assessed and emotional behavior during alcohol abstinence was evaluated.

Internal desynchronization in female rats does not affect alcohol consumption but alters scores of emotionality during alcohol abstinence. Changes in affective-like behaviors were accompanied by reduced body weight gain and estrous irregularities under aberrant LD conditions. Our data suggest that internal desynchronization caused by environmental factors is not a major factor contributing to the onset and progression of alcohol abuse, but highlights the need to maintain circadian hygiene as a supportive remedy during alcohol rehabilitation.

1.2 Introduction

Alcohol abuse is a widespread public health concern with adverse social and economic ramifications. Individuals with alcohol use disorder (AUD) have impaired control over drinking and continue to drink despite deleterious consequences to their health and professional lives (Rehm et al., 2009). AUD affects over 280 million people worldwide (Glantz et al., 2020), including more than 18% of the Canadian population (Statistics Canada, 2013). Clinical studies indicate that workers with night and rotating working schedules are more likely to suffer from binge drinking disorders compared to daytime workers (reviewed by Richter et al., 2021).

Although the causes of excessive alcohol consumption are multifactorial, including genetic, socio-economical, and psychological factors, growing evidence points to the important role of the internal time-keeping system in the development and progression of alcohol abuse (Barko et al., 2019; Parekh et al., 2015; Partonen, 2015). The circadian system refers to the hierarchical coordination of biological clocks located in the brain and peripheral tissues and organs that control daily rhythms in physiology and behavior. These biological timers rely on so-called clock genes and their protein products to generate 24-h rhythms and their synchronization to environmental periodicities such as the solar cycle. Genetic studies in humans and rodents revealed the influence of circadian clock gene modification on excessive alcohol consumption and abuse (Kovanen et al., 2010; Spanagel, Pendyala, et al., 2005). In addition, disruption of circadian rhythms has been shown to alter alcohol consumption, which is associated with differential accumulation of ΔFosB in brain regions related to the control of alcohol consumption (Reséndiz-Flores & Escobar, 2019).

Nevertheless, circadian rhythm disruption affects not only the reward-related neural circuitry but also contributes to stress-related mood disorders. Clinical studies have linked clock gene variants with depressive and anxiety-like behavior in humans (reviewed by Partonen, 2012). Moreover, rodents exposed to constant light or continuous alterations in LD cycles not only increase alcohol consumption but also promote the development of anxiety and depression-like behavior (Clark et al., 2007; Rosenwasser et al., 2010, 2015). Indeed, alcohol misuse is highly comorbid with neuropsychiatric disorders including anxiety and depression, and diagnosed patients have poorer alcohol rehabilitation outcomes, and a higher probability of relapse (Heilig et al., 2010; Lynskey, 1998). The intensity and context of craving to consume alcohol stem from emotional discomfort

during alcohol abstinence which may explain an increased relapse risk (Karpyak et al., 2016; Koob & Volkow, 2016).

Although not fully understood, excessive alcohol consumption and recovery may be driven, in part, by sex-specific neurobiological mechanisms (Becker & Koob, 2016; McHugh et al., 2018). While men were more often diagnosed with AUD than women, recent evidence points to a substantial increase in alcohol consumption among women, closing the gender gap in alcohol abuse and dependence (Ait-Daoud et al., 2017; McKetta & Keyes, 2019). AUD-diagnosed women are more likely to display comorbid affective symptoms compared to men (Abulseoud et al., 2013; Holzhauer et al., 2019). In particular, negative emotions are stressful for women, which cues more alcohol reinforcement and craving than in men (Palma-Álvarez et al., 2019; Peltier et al., 2019). Thus, negative affect and stress play a pivotal role in the development of addiction, including initiation and relapse, in particular for women (Koob, 2009).

Even though there is emerging evidence that chronodisruption affects alcohol drinking behavior differently in males and females (Rizk et al., 2022), only little is known about the sex-specific consequences of chronodisruption on mood and their interaction in the development of AUD. The relationship between circadian rhythm disruption and changes in reward and mood-related behavior remains poorly understood. Besides the utmost importance of the female sex in alcohol studies, research mostly focuses on clinical male cohorts or male rodents, leaving implications for the female sex in doubt (Zucker & Beery, 2010). To address this, we used female rats to investigate the effects of internal desynchrony on binge-drinking-like behavior, and emotion-related behavior during alcohol abstinence.

Our findings demonstrate that internal desynchronization has only minor effects on alcohol-drinking behavior in female rats but amplifies emotionality during alcohol abstinence. This study represents an important step in understanding the effects of circadian disruption on behavioral and physiological processes connected to reward and mood in the female sex.

1.3 Materials and methods

1.3.1 Animals and housing

Adult female Wistar rats (age 95 - 120 days, Charles River Laboratories, Canada) were kept individually in transparent cages equipped with running wheels (Tecniplast, Italy) in light (standardized illuminance of ~ 200 - 300 lux during the light period, 0 lux during the dark) and temperature- and humidity controlled (temperature: 21 ± 1 ºC, relative humidity: 65 ± 5 %) rooms. All animals had access to food and water *ad libitum*. Only animals with a regular estrous cycle were included in the study. Food consumption and body weight were monitored once a week throughout the experiment. Prior to the experiment, rats were kept under a standard 12:12 hour light-dark (LD24) schedule for three weeks. Following acclimatization, all animals were randomly assigned to be kept under standard (CTRL) or desynchronizing lighting conditions. We used two different light-dark (LD) paradigms to induce circadian desynchrony: A chronic phase shift schedule (SHIFT), consisting of 6-hour advanced shifts in the onset of the light phase every second day, by shortening the dark phase (Casiraghi et al., 2012), and a short-day 11:11 hour light-dark (LD22) paradigm, shown to induce desynchronization between the two oscillators in the core and shell of the suprachiasmatic nucleus (SCN) (De La Iglesia et al., 2004).

1.3.2 Alcohol drinking paradigm

To induce voluntary alcohol consumption in rats, the intermittent alcohol exposure (IAE20%) paradigm was used (Wise, 1973). The IAE20% drinking paradigm resulted in an efficacious model for inducing high levels of voluntary alcohol consumption in several rat strains (Mill et al., 2013; Simms et al., 2008; Wise, 1973). The protocol represents an alcohol binge-drinking-like pattern by alternating alcohol access and deprivation periods. Alcohol-naïve rats were given access to 20% ethanol solution (v/v) and tap water (two-bottle choice) for three 24-hour sessions per week (Monday, Wednesday, and Friday). On alcohol-free days (Tuesdays, Thursdays, Saturdays, and Sundays), rats received water only. Fluid bottles were weighed and replaced daily at ZT4 (ZT- Zeitgeber time; ZT0 represents the time of light onset). Bottles were weighed before and after the replacement in all groups. To control for side preferences, the placement of the bottles was alternated during the alcohol-drinking session.

1.4 Behavioral tests

1.4.1 Sucrose preference test (SPT)

Reward-based anhedonia-like behavior was assessed by monitoring sucrose intake (Eagle et al., 2016). First, rats were habituated to two bottles of 1% sucrose solution (m/v) for 48 hours to avoid neophobia. Subsequently, animals underwent food and water deprivation for 20 hours. In the final SPT, all animals had access to one bottle of 1% sucrose solution and one bottle of water for three hours. The total amount of sucrose solution and water intake during this period was recorded. All groups were tested at ZT2. Sucrose preference was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake.

1.4.2 Open field test (OFT)

The OFT was performed to evaluate exploratory and anxiety-like behavior (Prut & Belzung, 2003). Prior to the test, animals were habituated to the testing room for 30 minutes. At the beginning of the test, each rat was placed at the left-back corner of the open field arena (39 x 42 x 50 cm, TruScan Photo beam Activity Monitors, Coulbourn Instruments, MA, USA). The animals were allowed to freely explore the arena for 5 minutes. The animals' location and movements were recorded by infrared beams and analyzed by the software TruScan 2.0 (Coulbourn Instruments, MA, USA). All animals were tested at ZT2.

1.4.3 Elevated plus-maze (EPM)

The EPM was used to assess anxiety-related behavior (Walf & Frye, 2007). The maze consists of two open arms and two closed arms (30 x 10 cm, respectively), elevated 50 cm above the floor. The test was performed at the beginning of the dark phase, 2 hours after the lights were turned off (ZT14). All animals were habituated to the room conditions for 30 minutes. To start the test, rats were placed in the middle of the intersection between the open and closed arms facing the open arms. Behavior was video-recorded for 5 minutes.

1.4.4 Marble burying task (MBT)

The MBT was used to evaluate impulsiveness, irritability, and compulsive behavior. Burying behavior of harmless objects such as marbles is suggested as a form of impulsive behavior (Schneider & Popi, 2007) or indicates high anxiety levels (Li et al., 2019). Twenty glass marbles (ø 1.5 cm) of different colors were evenly spaced in five rows of four in a transparent plexiglass cage measuring 34 x 54 cm, filled with Sani Chips bedding (Teklad, Envigo, 5cm). At ZT2, rats

were placed individually in the cages and were allowed to explore the cages for 30 minutes. The number of buried marbles was counted at the end of the 30 minutes. Marbles were considered buried if two-thirds of the marble was covered with bedding.

1.5 Experimental procedures

1.5.1 Experiment 1 - Effect of different LD schedules and alcohol consumption on circadian disruption

In the first experiment, we evaluated the impact of various light conditions and alcohol consumption on circadian rhythmicity (Figure 1). Internal desynchronization was assessed by recording the locomotor activity under LD24 (n = 12), SHIFT (n = 6), and LD22 (n = 4) independently and in combination with alcohol consumption, deprivation, and re-introduction of alcohol (LD24 n = 12; SHIFT n = 12; LD22 n = 12). The severity of chronodisruption was assessed using the following circadian parameters: daily fragmentation (Intradaily Variability - IV), day-to-day stability (Interdaily Stability - IS), and the difference between day and night activity levels (Relative Amplitude - RA).

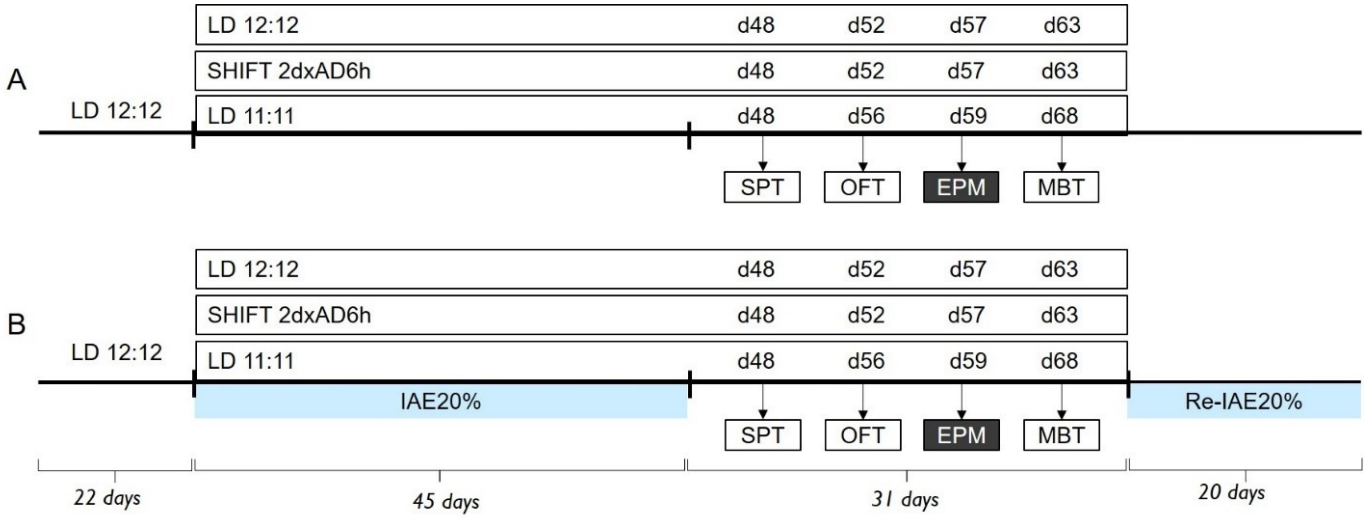


Figure 1 Experimental Design. Timeline of alcohol exposure and behavioral assessment (A) under LD24 (n = 12), SHIFT (n = 6), LD22 (n = 4) conditions, and (B) with access to intermittent alcohol exposure (LD24 n= 12, SHIFT n = 12, LD22 n = 12). Behavior tests were conducted during the light phase (ZT2), except for the EPM (ZT14). Zeitgeber (ZT) indicates hours after the onset of the light phase.

1.5.2 Experiment 2 - Effect of different LD schedules on alcohol consumption

This experiment determines if rats kept under artificial LD conditions will voluntarily consume more alcohol in a two-bottle choice paradigm compared to animals under standard LD24 conditions. Animals were kept either under LD24, SHIFT, or LD22 conditions and given intermittent access to alcohol for 20 sessions (Figure 1-B). We assessed alcohol preference (%) over water and alcohol intake (g /kg /24hr), as well as the relationship between alcohol preference and the animals' circadian rhythmicity under alcohol exposure.

Following alcohol access, alcohol was withheld to determine if high alcohol consumption levels would be maintained following a prolonged abstinence period. Thereafter, the animals were given again intermittent access to 20% alcohol for 10 alcohol-drinking sessions as described above. Comparisons were made between the mean alcohol consumption and alcohol preference, respectively, during the 10 alcohol-drinking sessions immediately before and after the abstinence period.

1.5.3 Experiment 3 - Effect of different LD schedules on emotional behavior during alcohol abstinence

We next compared the behavioral performance of animals kept under LD alterations alone and LD alterations during alcohol abstinence in a variety of behavioral tests (Figure 1). In the alcohol groups, behavioral tests were conducted 24 hours after the last alcohol access, under alcohol-free conditions. Animals followed the same testing order, starting with SPT, followed by the OFT, EPM, and MBT. The differences in the testing days of SHIFT and LD22 are due to the external LD schedule to test animals at the same zeitgeber time. Animals had a period of three to five days to recover between tests.

1.5.4 Experiment 4 - Effect of different LD schedules during alcohol access and abstinence on body weight and estrous regularity

We determined the effect of chronic LD alterations on body weight without and with alcohol access and deprivation. Relative changes in body weight were calculated based on the individual body weight at the beginning of the experiment. As stress and artificial light conditions influence the female estrous regularity, we characterized the estrus cycle over 24 days (24 days as a multiplicand of a 4-day estrous cycle) of each LD paradigm and alcohol condition.

1.6 Data Analysis

We quantified circadian rhythmicity by continuous registration of locomotor activity using running wheels. Summed wheel revolutions were recorded and stored over 10-minute intervals using the VitalView system (VitalView, Starr Life Science, USA). Locomotor activity data from the last 14 days of each LD condition and phase of alcohol exposure were used for the Chi-square-periodogram and circadian parameter analysis. Chi-square-periodograms (Refinetti, 1992; Sokolove & Bushell, 1978) were compiled to estimate the period of the daily activity rhythms under the various LD conditions (LD24, SHIFT, LD22) and in combination with alcohol access and abstinence using ClockLab 6 (Actimetrics Software, United States). Results were reported as Chi-square amplitude. To obtain additional measures of activity disruption we assessed RA, IV, and IS based on the equations reviewed by Brown (2019) using ClockLab 6 (Actimetrics Software, United States) for the last 14 days of each LD and alcohol accessibility phase. Wheel running activities were visualized as double-plotted actograms using ClockLab 6 (Actimetrics Software, United States).

Statistical analysis was performed using the GraphPad Prism 9 software package (GraphPad, San Diego, USA). All results were presented as mean ± standard error of the mean (SEM) and were examined for normality and homogeneity of variance. The significance level was set at $p < 0.05$. A one-way ANOVA (LD effect) was used to compare the rhythm parameters IV, IS and RA between the LD regimens, followed by a Dunnett's post-hoc test if a significant main effect was found. Further evaluation of the effect of the light regimen or alcohol exposure on circadian parameters during periods of alcohol access or abstinence was conducted by a two-way ANOVA (LD, alcohol effect) and Dunnett's post-hoc comparison.

Alcohol consumption and preference, as well as water consumption between the animals, kept under different LD conditions over the 20 exposure sessions, were assessed by a two-way repeated measures ANOVA (LD and time effect). The effect of LD conditions and progression of alcohol exposure on alcohol intake and preference before and after alcohol abstinence was assessed in a two-way repeated measure ANOVA.

Behavioral tests were analyzed using two-way ANOVA (LD effect, alcohol effect), followed by Šídák's post hoc analysis when a significant overall effect was found.

A two-way ANOVA was conducted to evaluate the effect of the different LD conditions and alcohol exposure on body weight.

The female estrous cyclicity was assessed by analyzing wheel running activity (Wollnik & Turek, 1988) over a period of 24 days within each alcohol availability phase under standard or altered LD conditions by chi-square analysis using the LSP software (www.circadian.org) (Sokolove & Bushell, 1978). This method provides a longitudinal, and non-invasive approach to avoid the risk of false-positive/negative results by inducing an irregular estrous cycle or the status of pseudopregnancy compared to the vaginal smears method (Cora et al., 2015). We characterized the estrous cyclicity by the number of rats showing a regular estrous cycle and evaluated the effects of LD conditions or alcohol using a two-way ANOVA.

1.7 Results

1.7.1 Experiment 1 - Effect of different LD schedules and alcohol consumption on circadian disruption

Exposure to non-24-hour LD cycles had a significant effect on daily activity rhythms (Figure 2, Figure 3). Internal desynchronization was observed in animals kept under SHIFT and LD22, indicated by the occurrence of two distinct activity peaks (Figure 2-A). Assessment of various rhythm parameters confirmed the deleterious effect of non-24h LD conditions on daily locomotor activity patterns (Figure 2-B). Whereas IV was not affected by the LD conditions ($F_{(2,17)}$= 0.04154, p= 0.9594, one-way ANOVA), a significant main effect of the LD conditions on IS ($F_{(2, 17)}$= 21.85, p< 0.0001, one-way ANOVA) and RA ($F_{(2,17)}$= 5.845, p= 0.0117, one-way ANOVA) was found. Both parameters were significantly reduced in animals exposed to non-24-hour light cycles.

When considering alcohol exposure as an additional factor besides the LD condition, the same rhythm parameters as mentioned above were significantly affected by both factors (Figure 4, Figure 5, Table 2). Interestingly, the effect of alcohol on IS and RA was primarily accompanied by the change in the LD condition, and the significant interaction underpinned this conclusion. In LD24 animals, there was no effect of the alcohol condition on the rhythm stability throughout the experiment (Figure 4-B). Moreover, no differences in IS and RA between alcohol exposure vs alcohol-free periods under chronically altered LD conditions (SHIFT or LD22) were found.

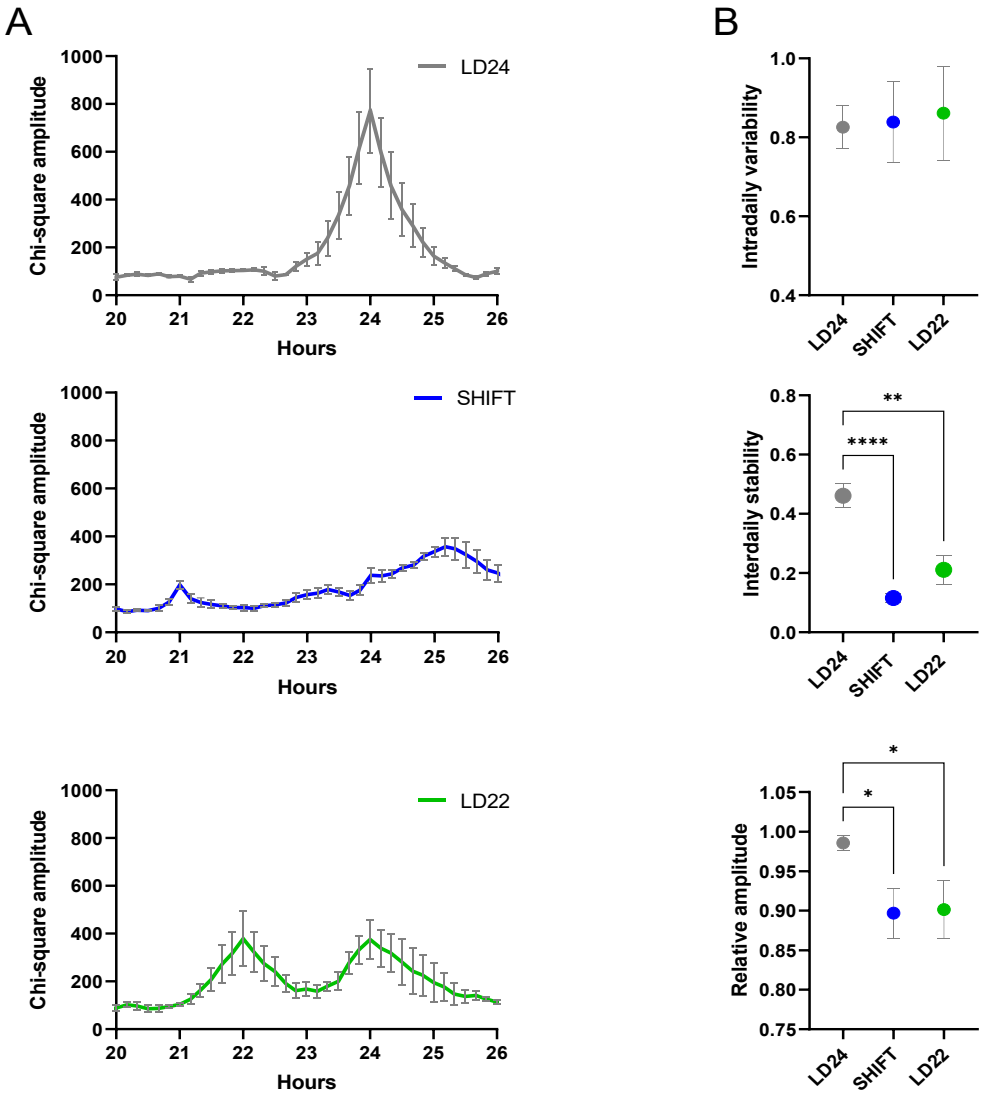


*Figure 2 Locomotor activity under aberrant LD schedules. (A) Comparison of periodograms and actograms under LD24, SHIFT, and LD22 conditions and (B) determination of Intradaily Variability (IV), Interdaily Stability (IS), and Relative Amplitude (RA) over 14 days. One-way ANOVA, Dunnett's multiple comparisons test: **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$; ±SEM; $n = 4 - 12$.*

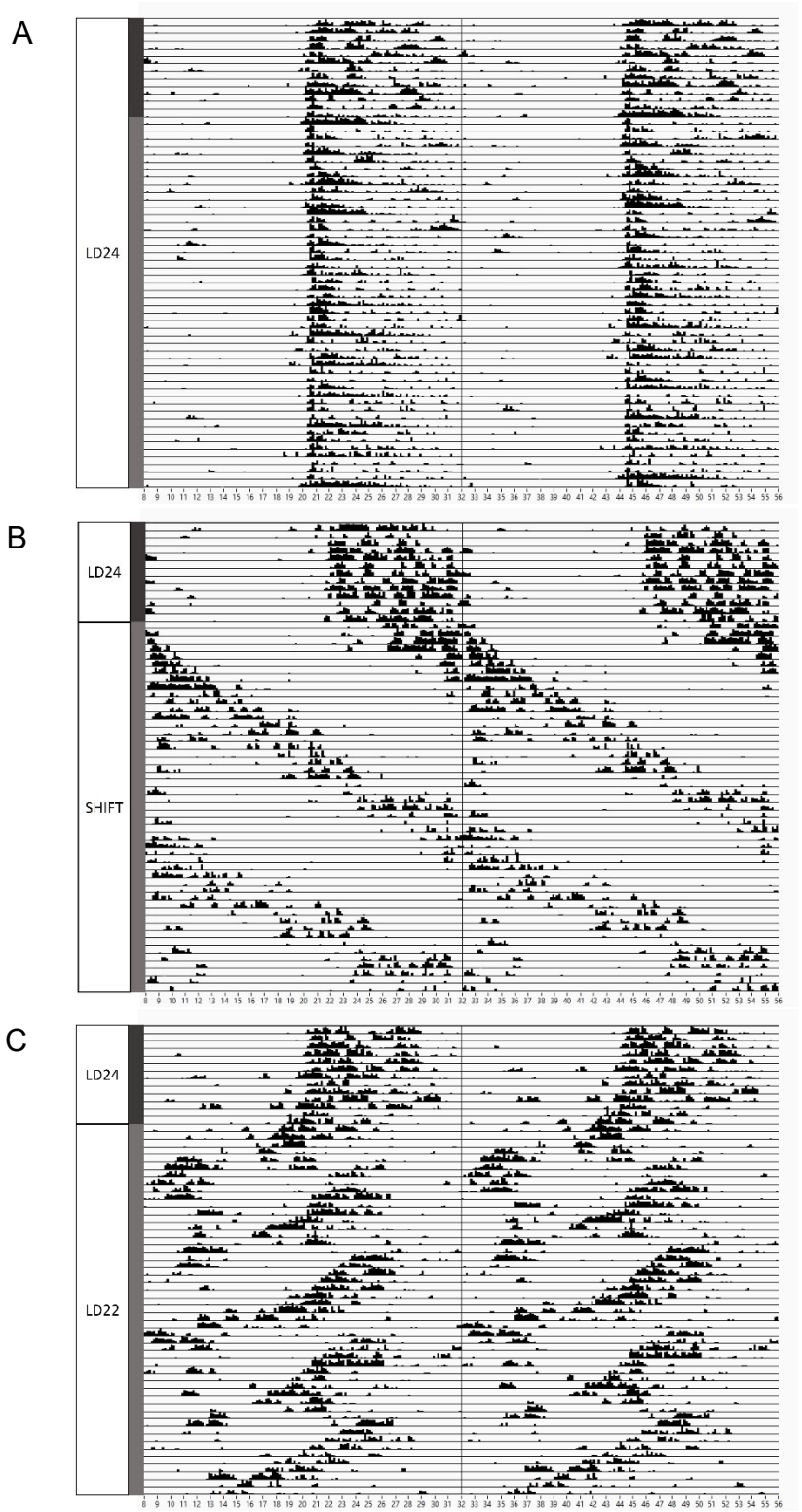


Figure 3 Representative circadian actograms of one animal kept under (A) LD24, (B) SHIFT, and (C) LD22 conditions over 50 days. Time of day is double-plotted along the x-axis, and successive days are arranged from top to bottom along the y-axis.

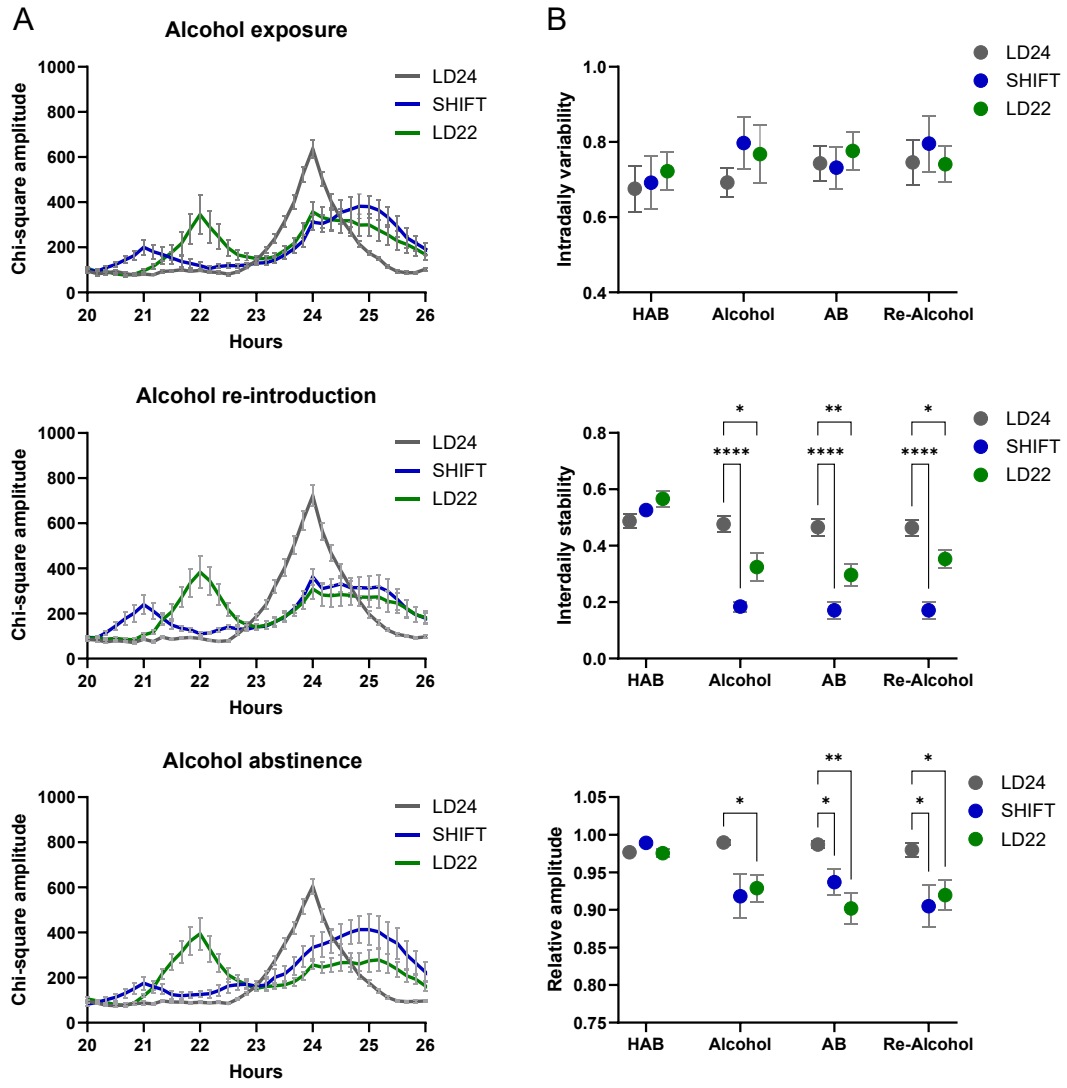
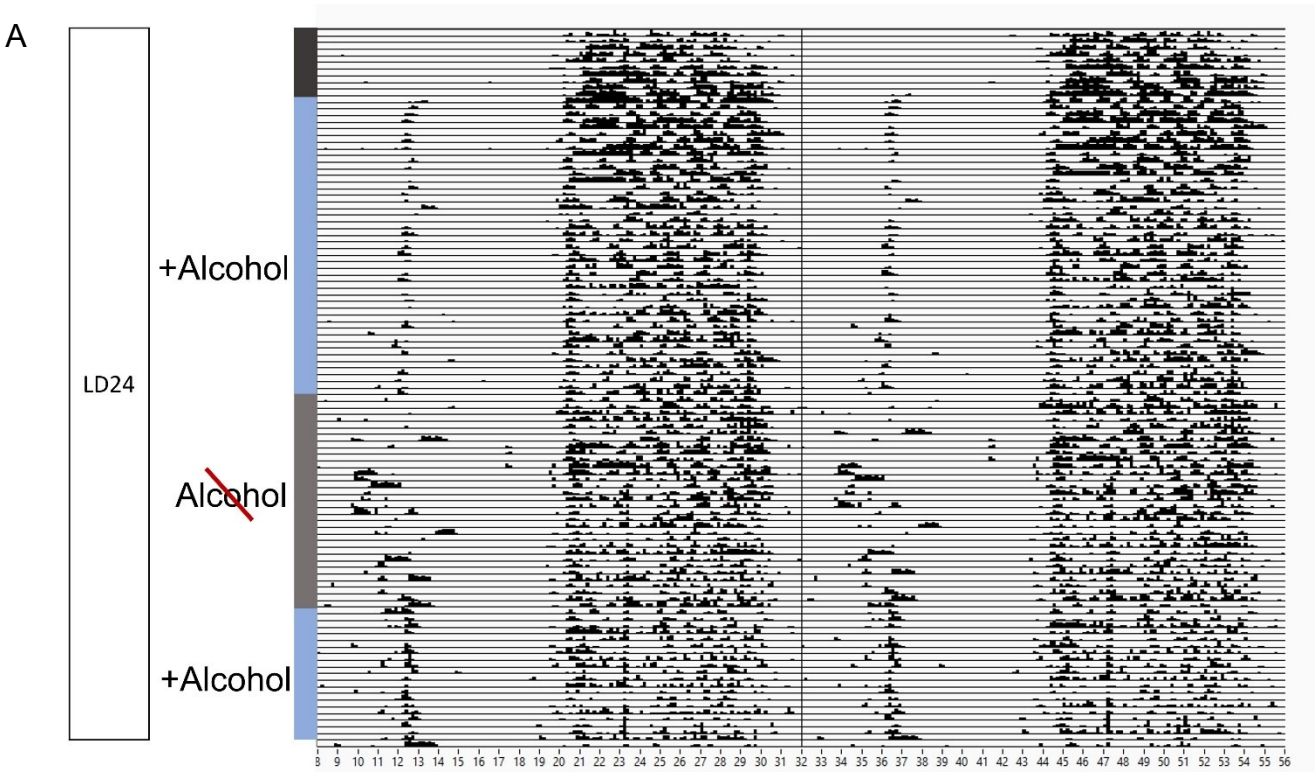
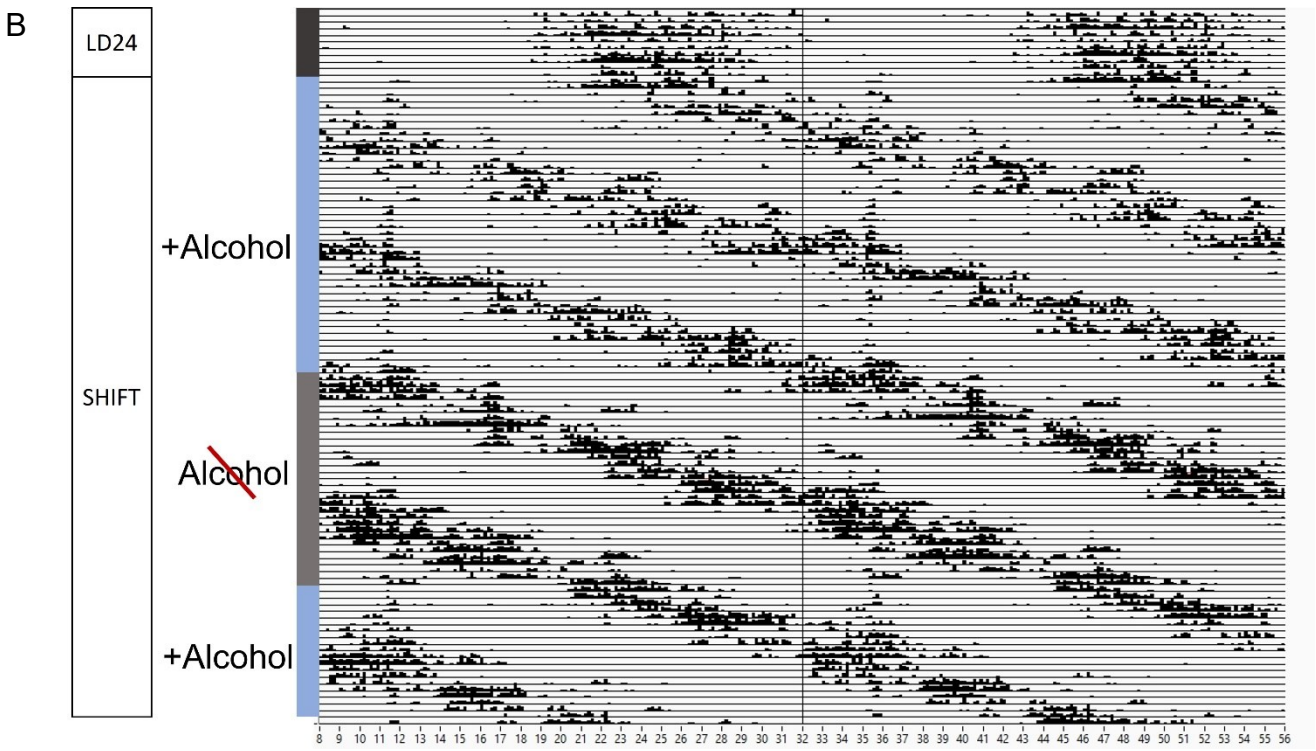


*Figure 4 Locomotor activity under altered LD conditions and intermittent alcohol exposure. (A) Comparison of periodograms and actograms during access to alcohol, alcohol abstinence, and re-introduction of alcohol. (B) Intradaily Variability (IV), Interdaily Stability (IS), and Relative Amplitude (RA) during habituation (HAB), intermittent access to 20% alcohol solution (Alcohol), abstinence (AB), and reintroduction of alcohol after abstinence (Re-Alcohol). Two-way ANOVA, Dunnett's multiple comparisons test: **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$; ±SEM; n = 4-12 /LD condition.*

Parameter	Two-way ANOVA		
	LD condition	**Alcohol condition**	**LD x Alcohol**
IV	$F_{(2, 33)} = 0.3046$, $p = 0.7792$.	$F_{(2.798, 92.34)} = 1.197$, $p = 0.2987$	$F_{(6, 99)} = 0.5810$, $p = 0.8264$
IS	$F_{(2, 33)} = 24.37$, $p < 0.0001$	$F_{(2.769, 91.38)} = 42.75$, $p < 0.0001$	$F_{(6, 99)} = 10.93$, $p < 0.0001$
RA	$F_{(2, 33)} = 7.754$, $p = 0.0017$	$F_{(2.729, 90.05)} = 5.378$, $p = 0.0026$	$F_{(6, 99)} = 2.479$, $p = 0.0282$

Table 1 Statistical results of circadian parameters for the combination of altered LD schedules and alcohol consumption.


A
LD24
+Alcohol
Alcohol
+Alcohol
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56


B
LD24
SHIFT
+Alcohol
Alcohol
+Alcohol
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56

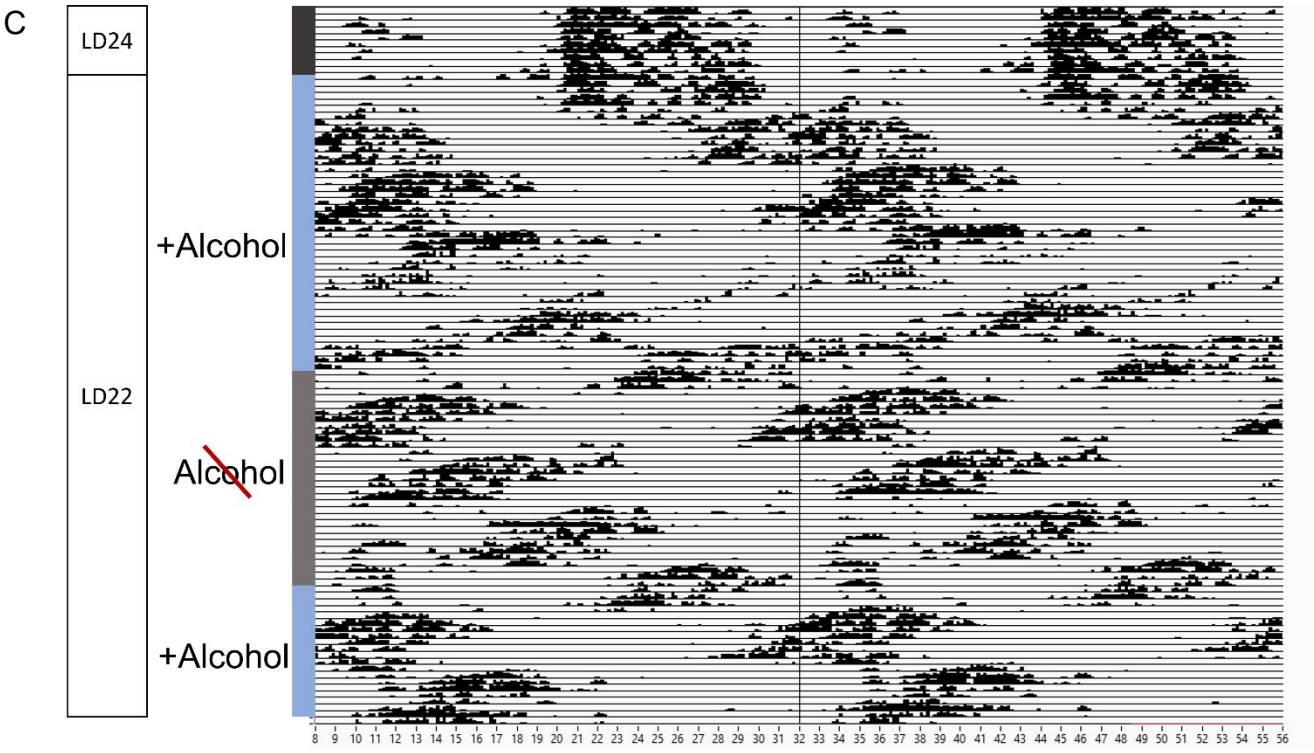


Figure 5 Representative circadian actograms of one animal kept under (A) LD24, (B) SHIFT, and (C) LD22 conditions during alcohol exposure and abstinence. Time of day is double-plotted along the x-axis, and successive days are arranged from top to bottom along the y-axis and labeled with the coinciding alcohol exposure phase.

1.7.2 Experiment 2 - Effect of different LD schedules on alcohol consumption

1.7.2.1 Experiment 2a - Effect of different LD schedules on intermittent alcohol consumption

No significant effect of the LD condition on alcohol intake, preference, and water intake was found (Figure 6, Table 2). A significant effect of the progression of the experiment on alcohol preference was observed, indicating that animals' preference for alcohol increases over time, irrespective of the LD conditions.

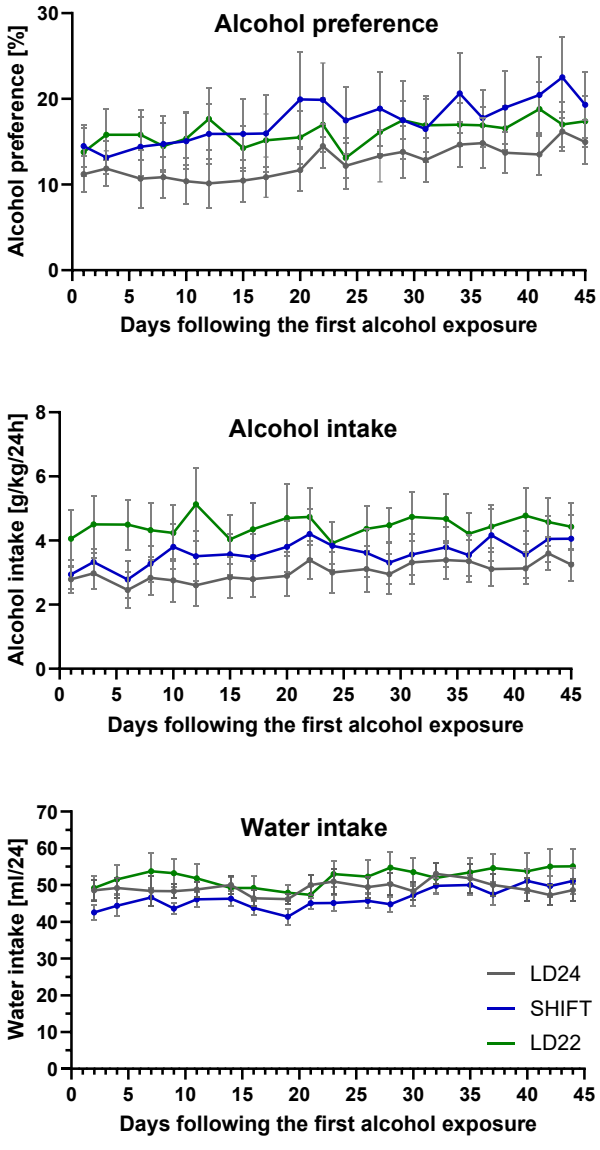


Figure 6 Time course for alcohol and water consumption in rats exposed to LD24, SHIFT, and LD22 paradigms. ±SEM; n = 12 /LD paradigm.

1.7.2.2 Experiment 2b - Effect of different LD schedules on a prolonged period of abstinence and subsequent alcohol consumption

After 20 sessions of voluntary alcohol consumption, animals from Experiment 2a were deprived of alcohol and re-exposed to alcohol after 31 days of abstinence. The LD conditions did not affect alcohol re-exposure, and no effect was found on either alcohol intake or preference. We did not observe an effect of sessions on alcohol drinking or preference and water consumption over 10 drinking sessions before and after prolonged alcohol abstinence either (Table 2).

Parameter	Two-way repeated measures ANOVA		
	LD condition	**Time**	**LD x Time**
Alcohol preference	$F_{(2, 33)} = 0.8352$, $p = 0.4428$	$F_{(6.717, 221.7)} = 2.883$, $p = 0.0075$	$F_{(38, 627)} = 0.5337$, $p = 0.9907$
Alcohol intake	$F_{(2, 33)} = 1.463$, $p = 0.2461$	$F_{(6.656, 219.6)} = 1.170$, $p = 0.3219$	$F_{(38, 627)} = 0.4835$, $p = 0.9965$
Water intake	$F_{(2, 33)} = 1.276$, $p = 0.2926$	$F_{(2.051, 67.68)} = 2.820$, $p = 0.0653$	$F_{(36, 594)} = 0.9108$, $p = 0.6206$
Abstinence effect: alcohol preference	$F_{(2, 33)} = 0.5746$, $p = 0.5684$	$F_{(1, 33)} = 3.948$, $p = 0.0553$	$F_{(2, 33)} = 0.1279$, $p = 0.8803$
Abstinence effect: alcohol intake	$F_{(2, 33)} = 1.086$, $p = 0.3494$	$F_{(1, 33)} = 2.178$, $p = 0.1495$	$F_{(2, 33)} = 0.01568$, $p = 0.9844$
Abstinence effect: water intake	$F_{(2, 33)} = 0.3169$, $p = 0.7306$	$F_{(1, 33)} = 0.08374$, $p = 0.7741$	$F_{(2, 33)} = 2.169$, $p = 0.1304$

Table 2 Statistical results of fluid consumption and the abstinence effect on fluid consumption.

1.7.3 Experiment 3 - Effect of different LD schedules on emotional behavior during alcohol abstinence

Emotional and reward-related behaviors were evaluated in animals exposed to standard (LD24) and artificial LD paradigms (SHIFT, LD22) during alcohol abstinence (Figure 7).

1.7.3.1 Sucrose preference test

A significant main effect of the LD conditions was found (Figure 7-A, Table 3), indicating that sucrose preference was altered under aberrant LD conditions. Subsequent post-hoc analysis unveiled a distinction between the groups with "SHIFT: no Alcohol" and "LD22: Alcohol abstinence".

1.7.3.2 Open field test

Rats show a strongly reduced exploratory activity in a novel environment during alcohol abstinence, irrespective of the environmental LD conditions. A significant main effect of the alcohol condition on the total distance traveled in the open field was found (Table 3). Post-hoc analysis revealed a significant decrease in locomotion during alcohol abstinence in all three experimental groups (Figure 7-B).

1.7.3.3 Elevated plus maze test

Desynchronizing light conditions affected anxiety-like behavior during abstinence in female rats. A significant main effect was found for both, LD and alcohol conditions (Table 3). The significant LD x alcohol interaction, however, indicates that the effect of the alcohol condition was not uniform across the different LD conditions (Figure 7-C, Table 3). Anxiety-like behavior is significantly increased during abstinence in rats kept under aberrant LD conditions when compared to rats kept under regular LD24 cycles (Figure 7-C).

1.7.3.4 Marble burying task

Similar to outcomes in the elevated plus maze, significant main effects for LD and alcohol condition, as well as a significant interaction between both factors, were found in the marble burying test (Figure 7-D, Table 3). A significant reduction in the number of buried marbles was found in rats kept under LD22 and SHIFT cycles compared to LD24 controls during abstinence-only (Figure 7-D).

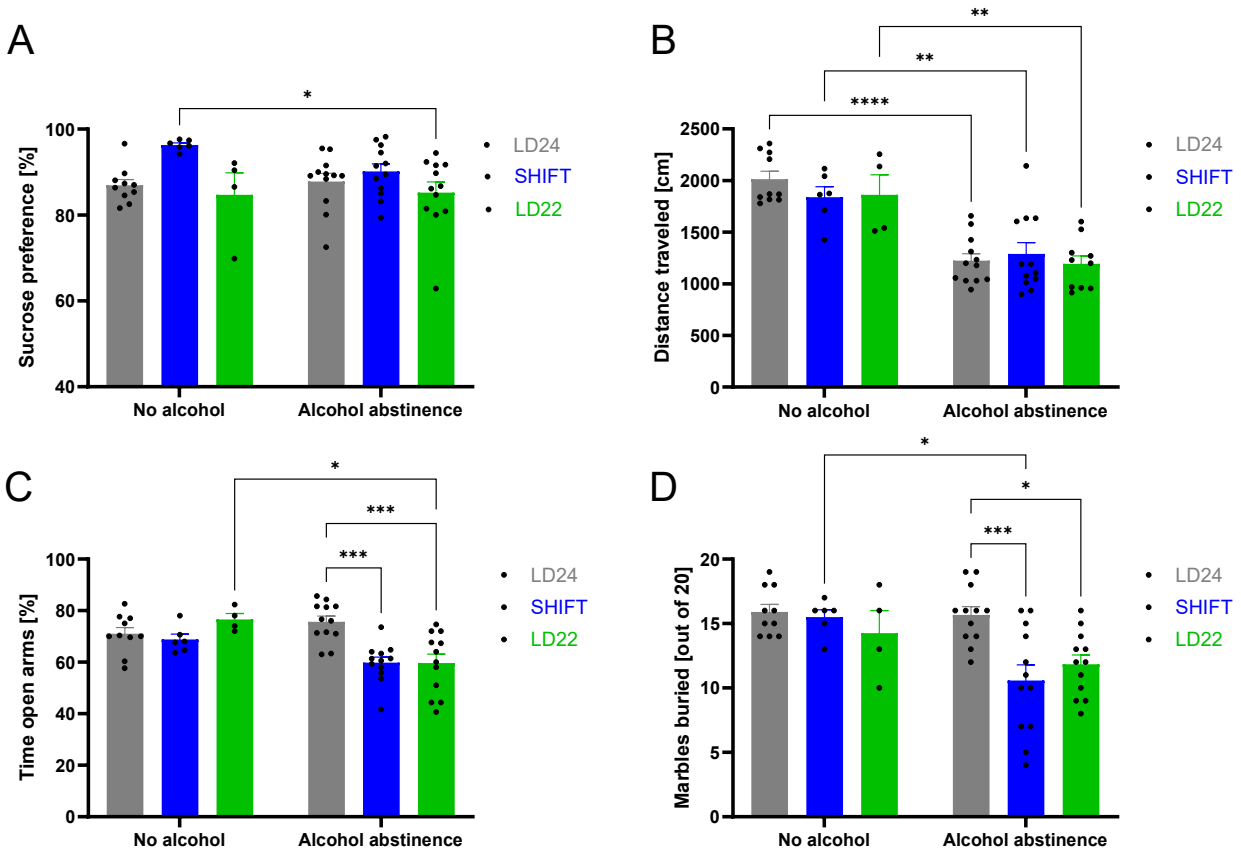


*Figure 7 Effect of LD schedule and alcohol abstinence on behavioral performance. (A) Sucrose preference (%) over a 3-hour test period, (B) distance traveled in the OFT, (C) the percentage of time spent in the open arms during the EPM, and (D) marbles buried within the MBT. Two-way ANOVA, Šídák's multiple comparisons test: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ±SEM; n = 4-12 /LD condition.*

Parameter	Two-way repeated measures ANOVA		
	LD condition	**Alcohol condition**	**LD x Alcohol**
Sucrose preference (SPT)	$F_{(2, 50)} = 6.269$, $p = 0.0037$	$F_{(1,50)} = 0.6940$, $p = 0.4088$	$F_{(2,50)} = 1.518$, $p = 0.2292$
Distance traveled (OFT)	$F_{(2, 48)} = 0.4203$, $p = 0.6592$	$F_{(1,48)} = 62.21$, $p < 0.0001$	$F_{(2, 48)} = 0.8106$, $p = 0.4506$
Time open arms (EPM)	$F_{(2, 50)} = 5.383$, $p = 0.0067$	$F_{(1,50)} = 8.233$, $p = 0.0060$	$F_{(2,50)} = 6.910$, $p = 0.0022$
Marbles buried (MBT)	$F_{(2, 50)} = 5.843$, $p = 0.0052$	$F_{(1,50)} = 9.468$, $p = 0.0034$	$F_{(2,50)} = 3.214$, $p = 0.0486$

Table 3 Statistical results of behavioral performances.

1.7.4 Experiment 4 - Effect of different LD schedules during alcohol access and abstinence on body weight and estrous regularity

All rats showed a progressive increase in body weight over time (Figure 8-A). Body weight gain (%) differed significantly between the groups depending on time and time x LD paradigm interaction, but not LD condition (Table 4). We also found a pronounced LD effect on the estrous cycle (Figure 8-B; LD effect $F_{(2, 4)} = 12.95$, $p = 0.0179$, two-way ANOVA). In contrast, chronic intermittent alcohol consumption alone did not alter the estrous cyclicity under standard LD conditions (Alcohol effect: $F_{(2, 4)} = 1.430$, $p = 0.3399$, two-way ANOVA).

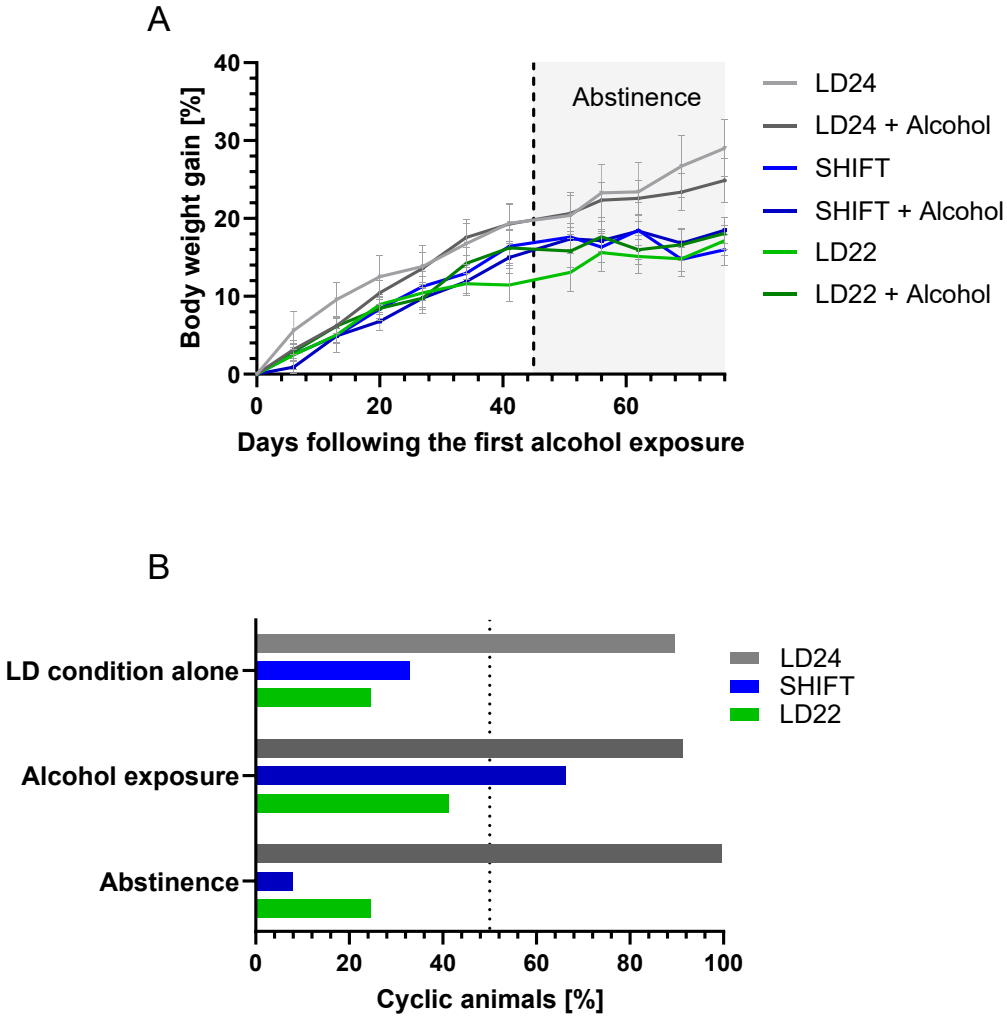


Figure 8 Body weight gain and estrous cyclicity under altered LD schedules, alcohol exposure, and abstinence. (A) Change in body weight gain (%) over time. (B) Comparison of estrous cyclicity between LD alone and in combination with alcohol access and abstinence as indicated by the percentage of animals showing a rhythmic estrous cycle (%). ±SEM; n = 4-12 /LD condition.

Parameter	Two-way repeated measures ANOVA		
	LD condition	Time	LD x Time
LD Alteration	$F_{(2, 17)} = 1.784$, $p = 0.1980$	$F_{(2.417, 41.08)} = 70.85$, $p < 0.0001$	$F_{(22, 187)} = 3.416$, $p < 0.0001$
LD Alteration + Alcohol	$F_{(2, 33)} = 2.174$, $p = 0.1298$	$F_{(2.945, 97.17)} = 109.3$, $p < 0.0001$	$F_{(22, 363)} = 1.477$, $p = 0.0780$

Table 4 Statistical results of body weight gain.

1.8 Discussion

The link between the disruptions of the circadian system and alcohol consumption has been recognized for decades. While most of the research focused on male cohorts, the effect of internal rhythm disruption on alcohol consumption in females is less known. We show that the induced internal desynchrony through chronic advanced shifts and LD22 persists throughout the course of alcohol intake and abstinence but is unaffected by the respective alcohol condition. Although aberrant light schedules, which simulate shiftwork and jet lag, tend to increase alcohol consumption and preference, we observe no significant differences between our experimental groups in female rats. Importantly, we demonstrated that internal desynchrony affects performance in tests of anxiety-like behavior during periods of alcohol absence. However, these behavioral alterations do not seem to predict or interact with alcohol consumption before abstinence or during re-exposure (data not shown). Taken together, our results demonstrate that internal circadian desynchronization strongly affects female physiology but has only minor effects on mood-related behavior and alcohol consumption if the two factors were considered separately. When combining the effects of alcohol consumption and circadian disruption, changes in mood-related behaviors became more apparent, which emphasizes the important contribution of dysregulation of circadian functioning in the etiology of mood-related behavior in females under alcohol abstinence.

1.8.1 Alcohol intake under different LD schedules

Experimental conditions of jet lag or constant, abnormal light conditions on alcohol consumption led to disparate results, depending on heterogeneity and breadth in approaches and concepts associated with circadian disruption. In our experiment, we observed similar alcohol intake (g/kg/ 24h) levels as previously reported in female Fischer, Lewis rats, and P rats (Rosenwasser et al., 2010, 2014) under standard LD conditions. Although the number of studies on chronodisruption in female rodent models is sparse, previous studies reported conflicting outcomes of chronodisruption on alcohol consumption in female rats (Clark et al., 2007;

Rosenwasser et al., 2010). Differences in the alcohol availability paradigm (continuous vs intermittent), alcohol concentration, and LD conditions may account for the different outcomes. Our results, however, are in line with studies conducted in mice that did not reveal differences in alcohol intake or preference in wild-type females exposed to an LD20 cycle compared to animals kept under standard LD conditions (Rizk et al., 2022). Overall, these outcomes indicate that aberrant LD conditions may not be a major factor contributing to abnormal alcohol-drinking behavior in female rodent models.

Numerous factors including species, sex, duration of aberrant light exposure, the direction of phase shift, and the time between shifts affect the independent and combined effects on behavioral and physiological variables (Vetter, 2018). However, studies often lack the quantification and validation of the success of internal rhythm disruption (Brown et al., 2019). In our study, we confirmed that the applied protocols of phase advances and LD22 conditions induce internal desynchrony in female rats, similarly as described previously in male rats (Ben-Hamo et al., 2016; Casiraghi et al., 2012). The degree of internal rhythm disruption, however, was not a strong predictor for alcohol consumption or preference (Chapter 2). Vice versa, alcohol consumption had no effect on circadian locomotor activity rhythms in female rats investigated in our study, which may be attributed to the use of running wheels. It is known from previous studies in rodents that running wheel activity affects the circadian system (Leise et al., 2013; Weinert et al., 2016; Weinert & Gubin, 2022) whereas contradicting effects were found on alcohol drinking behavior in rodent models, depending on species, sex, and drinking paradigm (Buhr et al., 2021; Ehringer et al., 2009; Ozburn et al., 2008; Piza-Palma et al., 2014). It is, however, conceivable that the independent effects of the running wheel on the circadian system and/ or alcohol drinking behavior mitigated the deleterious effects of the aberrant LD conditions and thus, affect alcohol intake and preference. The sex-specific effect of exercise on alcohol drinking should therefore be emphasized in future work.

Several studies indicate that molecular components of the circadian clock play a crucial role in the control of alcohol-drinking behavior (Ozburn et al., 2013; Spanagel, Pendyala, et al., 2005) and suggest that the impact of genetic components may even prevail over environmental influences (Rizk et al., 2022). However, the effects of a targeted deletion or downregulation of clock genes are not directly comparable to the effects of chronodisruption. Latter one leaves the

clock genes functionally intact but dysregulates in their daily expression, which has been demonstrated in brain areas associated with reward regulation and drug-related behaviors (Tamura et al., 2021). Nevertheless, core clock genes are extensively involved in regulating the dopaminergic reward circuitry, and disruption of those processes may increase the vulnerability to developing drug addiction (for review Depoy et al., 2017). Since dopamine transporters and norepinephrine receptors are expressed rhythmically, chronodisruption may dysregulate circadian patterns of neurotransmission and thus alter alcohol exposure (Sleipness et al., 2007). The exact role of circadian clock gene expression in brain regions associated with reward-related processes in female rodents exposed to aberrant LD conditions, however, should be investigated in follow-up studies.

Lastly, peripheral oscillators may impact the reward circuit in addition to the central clock. The release of sex hormones like estradiol is controlled by the circadian system (Barbacka-Surowiak et al., 2003) and the loss of internal rhythmicity affects the pulsatile hormone release (Sciarra et al., 2020). Importantly, sex hormones modulate neuronal activity and can influence alcohol-drinking behavior (Erol et al., 2019). It was shown that the artificial administration of estrogen increases alcohol intake and drug-seeking behaviors in females and males, although fluctuating sex hormone levels during the course of the estrous cycle do not markedly affect alcohol intake (Priddy et al., 2017). In our experiment, internally desynchronized animals show impaired estrous regularity, which may impose altered levels of sex hormones. Altered LD schedules may not only affect the sex hormone function directly but may also act as a chronobiological stressor (Boulos & Rosenwasser, 2004). It is known that stress can affect estrous regularity with consequences on hormone levels including corticosterone and estradiol as well as on the expression of sex hormone receptors (An et al., 2020). Likewise, excessive alcohol consumption disrupts the menstrual cycle in women (Lyngsø et al., 2014) and the estrous cycle in animals (Emanuele et al., 2002). However, similar to Satta (2018), we did not observe an effect of alcohol alone on the estrous cycle regularity in our study. Only in the combination of alcohol consumption with chronic phase advanced shifts markedly impaired estrous regularity. This suggests that internal desynchronization affects regular estrous functioning to a greater extent than alcohol consumption. Reports from female shift workers (Cai et al., 2019; Labyak et al., 2002), and studies in animals kept under artificial LD conditions support this view (Hardy, 1970; Yoshinaka et al., 2017). Future studies in ovariectomized individuals may further decipher the

mutual interactions of chronodisruption, estrous cycle, sex hormone fluctuations, and alcohol consumption.

1.8.2 Emotional behavior during alcohol abstinence

Extensive literature supports the association between circadian rhythm disruption and the emergence of anxiety and depression-like symptoms (Bedrosian & Nelson, 2017). Since negative emotional states may accelerate alcohol consumption and trigger a relapse, the association between circadian disruption and affective disorder is important in the context of the development and progression of aberrant alcohol-drinking behavior (Yang et al., 2018). In the present study, we used multiple behavioral measures to examine the emotional state changes of female rats during alcohol abstinence in the context of circadian disruption. As the time point for behavioral assessment is critical for the results and evaluation of behavior (Saré et al., 2021), we assessed anxiety-related behavior at two different points depending on the test (Nakano et al., 2016; Schoettner et al., 2022). Animals were tested at the same external time in correspondence to the LD conditions (Zeitgeber time). However, chronodisruption causes misalignments between internal and external circadian time. Thus, the internal circadian time of testing may vary across individuals within and between our experimental groups, despite testing at the same "external time". Testing at various time points using the same animal may be beneficial in this regard, but difficult to achieve in common tests for anxiety-like behavior like the elevated-plus maze because of the well-described "one-time tolerance phenomenon" of those behavioral tests in rodents (File et al., 1990; Holmes & Rodgers, 1998, 1999; Rodgers et al., 1992). Alternatively, when measuring behavior using the internal time (e.g. onset of locomotor activity rhythm) as a reference, it may not correspond to the same external time across chronodisrupted animals, and the test may thus be affected by external conditions such as light. The uniform behavioral responses in our chronodisrupted animals, however, indicate that behavioral alterations occurred independently of external or internal time.

Reduced exploratory behavior and increased anxiety-like behavior in rats under alcohol withdrawal have been demonstrated in the past (Overstreet et al., 2003; Rasmussen et al., 2006; Valdez et al., 2002). In line with these studies, we observed reduced locomotion in the OFT under alcohol abstinence, demonstrating the deleterious effect of previous alcohol consumption on anxiety-related behavior. In contrast, anxiety-related behavior in the EPM female rats under

conditions of alcohol abstinence was only observed in chronodisrupted animals. Previous work in male rats indicated that altered LD conditions alone induce anxiety-like behavior (Horsey et al., 2020; Okuliarova et al., 2016), which was not replicated in our study. The unequivocal response across anxiety tests indicated that the deleterious effects of chronodisruption and alcohol exposure and mood need to be considered for specific anxiety traits rather than generalized anxiety-like behavior. Moreover, sex, stress, or the use of running wheels may have mitigated the apparent effects of aberrant LD conditions on affective behaviors, depending on the behavioral test (Bilu et al., 2022; Binder et al., 2004; Novak et al., 2012; Sandi et al., 2008). Studies indicate that acute and chronic alcohol consumption modulates the expression and rhythmic pattern of circadian clock genes and affects various components of the stress hypothalamic-pituitary-adrenal (HPA)-axis (Perreau-Lenz & Spanagel, 2015). The combined aberrant effects of chronodisruption and alcohol consumption on the HPA axis may represent a central component in the control of mood-related alterations and should be investigated in more detail in future studies.

Increased reactive behavior during alcohol abstinence was not evident in the marble burying task. In our experiment, alcohol-experienced rats exhibited decreased burying behavior, whereas previous studies have shown that female rodents enhance marble-burying behavior during alcohol abstinence (Chavez et al., 2020; Umathe et al., 2008). As chronic phase shifts, alcohol binge drinking, and withdrawal have broad effects on cognition, memory function, and motivation, the ability to bury marbles might be even more compromised in the combination of all these (Ji et al., 2018; Stevenson et al., 2009). While the MBT is a widely accepted model to study anxiety and compulsive-like traits in rodents, findings depend strongly on contextually valid experimental design, and individual characteristics including cognitive function (de Brouwer et al., 2019).

Reduced sucrose consumption is often used as a measure of anhedonia to assess depressive-like traits in rodents (Liu et al., 2018). Studies exposing male rats to various LD schedules alone reported an increased depressive-like behavior associated with decreased sugar preference (Ben-Hamo et al., 2016; Horsey et al., 2020). We did not observe this behavior in alcohol-naïve female rats in our experiment. Factors including the experimental testing design for sugar preference (eg. length and exposure of the sugar solution), as well as the female sex, may be

accountable for the different outcomes. Similarly, alcohol-experienced animals displayed no differences in sucrose preference. These results are in line with studies that reported a decreased or no change in sucrose preference during alcohol abstinence in female rodents (Li et al., 2019; Metten et al., 2018; Pang et al., 2013).

1.9 Conclusion

Our study provides a foundation to understand how circadian desynchronization affects emotional behavior in female rats under alcohol abstinence. The female organism consists of a unique sex-dependent neurobiological, chronobiological, and hormonal setting, that distinctly reacts to chronic light-dark changes. In future studies, it will be important to distinguish between operational and acute sex-hormonal factors mediating the consequences of internal desynchronization and the effects of alcohol intake in females. A more comprehensive understanding of the role of sex-dependent circadian risk factors will be needed to provide sex-specific alcohol rehabilitation treatments incorporating affective disorders as an important factor in individuals performing under shiftwork conditions.

1.10 Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.11 Ethics statement

All animal-related experimental protocols and procedures were approved by the Canadian Council on Animal Care and the Concordia University Animal Care Committee (certificate number: 30000256).

Chapter 2: Exploring the relationship between circadian disruption, fluid consumption, and behavioral outcomes

Christiane Meyer, Konrad Schoettner, Shimon Amir

Author Contributions

CM and SA conceived and designed the experiments. CM conducted the experiments and was assisted by KS. CM analyzed and interpreted the data with input from KS and SA. CM did the data visualization. CM wrote the manuscript and revised by SA and KS. SA supervised the project.

2.1 Abstract

The study of rodent behavior has been instrumental in advancing our understanding and treatment of neuropsychiatric disorders, but there has been limited focus on the underlying neurophysiological mechanisms contributing to innate differences in behavioral performance. Chronodisruption has been identified as a determinant of alcohol misuse behavior, with concurrent mood-related symptoms suggesting shared pathophysiological mechanisms among chronodisruption, alcohol misuse, and mood. Thus, alterations in one measure may predict changes in other behavioral measures.

The primary objective of this study was to investigate the relationship between circadian disruption, alcohol consumption, and affective behavior in female rats. Our findings indicated no correlation between the degree of internal disruption and alcohol consumption. While we observed certain correlations between circadian disruption and behavioral outcomes, there were no significant associations found between circadian disruption and alcohol consumption, as well as alcohol consumption and behavior.

These results suggest that while manipulating light-dark conditions and alcohol intake appear to be related to observed behavioral changes, they do not account for all the observed variations. It implies that behavioral outcomes are strongly influenced by various external and individual factors that contribute to our findings. Therefore, it is crucial to acknowledge that the relationship between manipulating variables and assessing subsequent behavior is likely to be more intricate and caution must be exercised when interpreting and discussing the validity of these behavioral datasets.

2.2 Introduction

Behavioral neuroscience involves the application of biological principles to investigate the underlying molecular and physiological mechanisms that regulate behavior in humans and animals. Likewise, the exploration of brain dysfunctions related to neurological and psychiatric disorders has greatly enhanced our understanding of various medical conditions and disorders, encompassing domains such as clinical psychology and psychopathology. Although animal models may not fully capture the complexity of mental disorders, they provide valuable insights into numerous neuropathological conditions (Markou et al., 2008).

Behavioral tests are commonly used to establish cause-effect relationships between experimental manipulations and abnormal behavioral phenotypes, with the goal of elucidating the underlying mechanisms of neurobehavioral disorders. Nevertheless, it is important to recognize the limitations of behavioral testing to ensure accurate interpretation and a comprehensive understanding of the results.

In scientific studies, behaviors are often categorized into broad domains depending on the neurobiological pathways aimed to be examined. However, the range of behavioral tests extends beyond a single domain, as some tests can encompass multiple parameters of behavior. For example, the sucrose preference test can be used as a measure of stress-induced anhedonia or the rewarding effect of addictive drugs (Liu et al., 2018). Additionally, relationships between behaviors assessed in different tests can reveal important insights when the outcomes are combined. Individual differences in sucrose consumption in rats were predictive of behavioral reactivity in the elevated plus maze, a test of anxiety-like behavior (Desousa et al., 1998). Thus, one aspect of behavior can intertwine with another, and exploring these connections can serve as a valuable tool for uncovering the brain functions and networks involved in various psychopathological conditions, ultimately providing a better understanding of the complex nature of brain disorders.

Relationship analyses are indeed valuable tools for identifying associations between investigated parameters. However, in the field of behavioral neurobiology, the application of these analyses, particularly correlation analysis, is relatively sparse. This gap in the literature leaves us with a limited understanding of the correlations between manipulated factors and the evaluated

behaviors. To address this limitation, our study aims to investigate the correlations between measures of circadian rhythmicity, affective behavior, and alcohol consumption.

In Chapter 1 of our study, we examined the impact of circadian disruption and alcohol consumption on affective behaviors, and interesting differences were observed depending on the specific light or alcohol condition. However, the precise nature of the relationship between these variables remains uncertain. To delve deeper into the intricate interplay between physiological disruptions and behavioral responses, we conducted a thorough analysis that employed correlation analysis to assess the relationships between circadian and behavioral parameters and regression analysis to explore potential causal relationships between these variables.

Our findings suggest a complex relationship between circadian disruption and behavioral output measures and point to the possibility of other factors contributing to the observed behavior. This investigation holds significance as it sheds light on the potential effects of disrupted circadian rhythms on fluid intake and behavioral performance. By elucidating these relationships, this analysis contributes to the understanding of how circadian disruptions may influence individuals' drinking behaviors and affective behavior subsequently.

2.3 Data Analysis

The used dataset comprises both circadian and behavioral data of each individual used in the experiment of Chapter 1. We explored the relationships between circadian disruption, characterized by relative amplitude (RA) and interdaily stability (IS), and alcohol consumption, as well as their influence on alcohol intake behavior and performance in mood-related tests.

Pearson correlation analyses were performed using GraphPad Prism 9 software (GraphPad, San Diego, USA). We investigated the correlation between the average circadian output parameters (IS and RA) during the 14-day alcohol access period and the average fluid intake over 10 exposure sessions. Furthermore, we examined the correlation between IS and RA during the 14-day alcohol abstinence period and the average behavioral outcomes measured in Chapter 1 (sucrose preference in the SPT, distance traveled in the OFT, percentage of time in the open arms in the EPM, and the number of marbles buried out of 20 in the MBT). Additionally, we analyzed the correlation between alcohol intake and preference in the 10 sessions before and after the alcohol abstinence period with the aforementioned behaviors.

Moreover, we conducted a simple linear regression analysis using GraphPad Prism 9 software. This involved fitting a linear model with RA or IS as the predictor variable (cause) and fluid consumption as the outcome variable (effect). Following the strength and significance of the resulting regression coefficients using Pearson's correlation coefficient (r) were assessed.

2.4 Results

2.4.1 Relationship between circadian disruption and fluid consumption

We assessed the relationship between circadian disruption as a function of RA and IS, and fluid consumption during the last ten alcohol exposure sessions (Figure 9).

We observed a significant correlation between RA and alcohol preference and -intake in animals kept under standard LD24 conditions, but not in chrono-disrupted animals. Our analysis did not reveal a relationship between rhythm stability within the day (IS) and fluid consumption regardless of the used LD condition. The indicators of rhythm stability were correlated to each other under chrono-disruptive LD schedules, but not standard LD24 conditions.

Since correlation analyses measure the strength and direction of the linear relationship between two variables, regression analysis examines the relationship between a dependent variable and one independent variable, considering the potential influence of other factors. We employed a regression analysis to investigate a potential causal relationship between circadian disruption and fluid intake (Figure 10). The results of the regression analysis indicated that circadian disruption, either characterized by RA or IS, although weak, might influenced alcohol consumption. Specifically, lower RA or IS values were associated with higher alcohol preference/intake. The effect was more severe when considering the amplitude between daytime and nighttime activity (RA) instead of the stability within the day (IS). IS showed a significant impact only under SHIFT for alcohol preference and LD22 for alcohol preference and intake. Lastly, rhythm stability and amplitude influenced water consumption, but only under LD24 conditions. Lower RA or IS values were associated with lower water consumption. We did not observe any effects of RA and IS on water consumption under experimental LD conditions.

Importantly, the regression analysis revealed a very weak Pearson index for all conditions. In essence, finding a weak regression that is statistically significant suggests that that particular exposure has an impact on the outcome variable, but that there are other important determinants

as well. Therefore, in the case of a regression analysis revealing a weak relationship but reaching statistical significance, it implies that the observed relationship is likely to be reliable and not simply due to chance, even though the strength of the relationship may be limited.

Taken together, although the predictor variables did not show a significant correlation individually, they may still contribute to explaining the variation in the dependent variable when combined in the regression analyses.

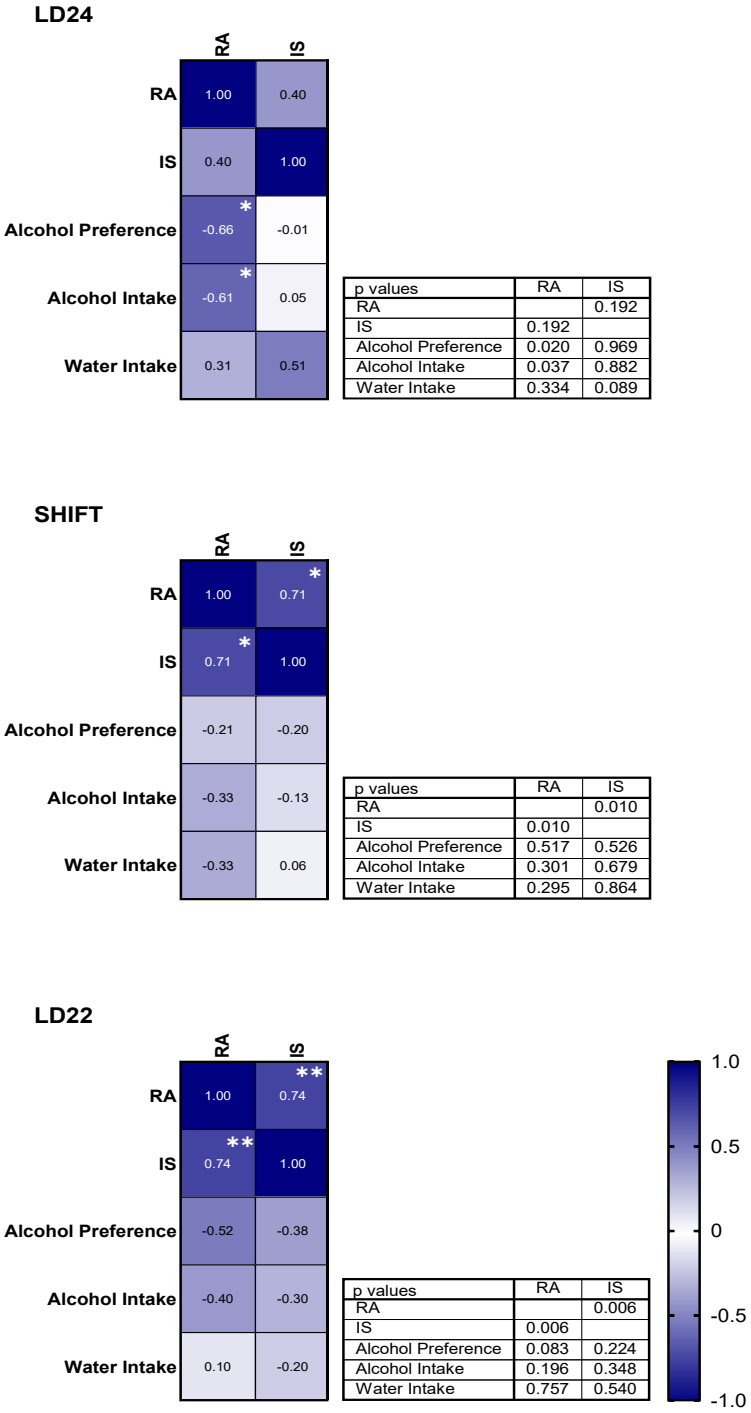


*Figure 9 Correlation analysis between circadian disruption and fluid consumption in rats exposed to LD24, SHIFT, and LD22 conditions. The graph visually presents the correlation coefficient (Pearson r), and the statistical results, including p-values, are displayed alongside their corresponding graphs. Significant values are indicated in the graphs with ** $p < 0.01$, * $p < 0.05$. ±SEM; n = 12 /LD paradigm.*

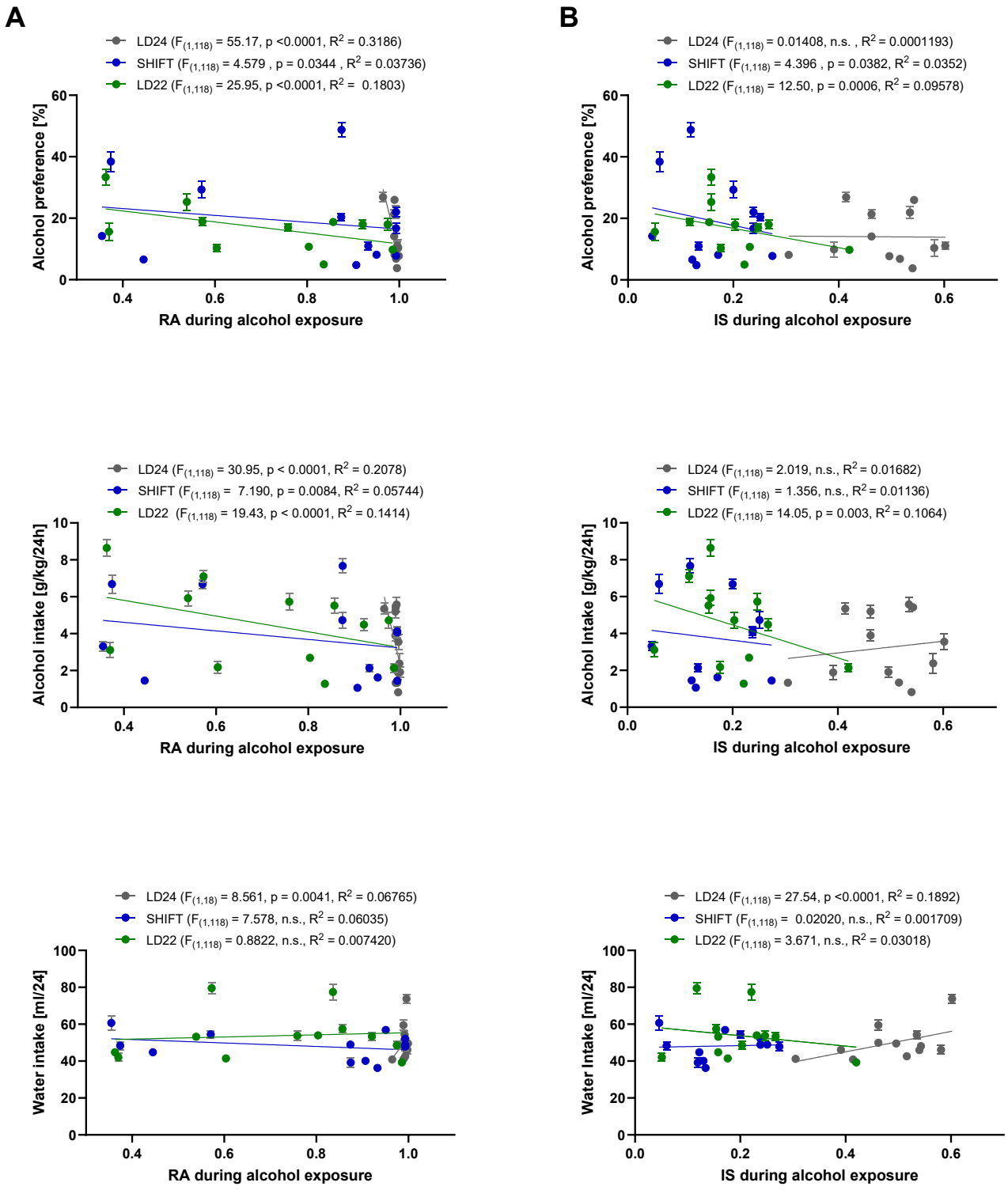


Figure 10 Individual distribution of animals exposed to LD24, SHIFT, and LD22 conditions and their fluid consumption. Regression analysis between circadian disruption as a function of the RA (A) and IS (B) and fluid consumption for the last ten alcohol exposure sessions. Statistical results of the regression analysis can be found at the top of each respective graph. ±SEM; n = 12 /LD paradigm.

2.4.2 Correlation between circadian disruption and behavioral phenotypes

Subsequently, we conducted a correlation analysis to examine the relationship between circadian disruption and behavioral performance during alcohol abstinence in various mood-related tests (refer to Chapter 1, Figure 11). If a significant correlation was observed, a regression analysis was carried out.

The analysis identified a significant correlation between RA or IS and SPT exclusively in animals kept under LD22 (Figure 11-A). A further conducted regression analysis confirmed a negative regression between RA or IS and SPT, with lower RA/IS predicting lower sucrose preference in the SPT (RA/SPT: $F_{(1,2)}= 66.39$, $p = 0.0147$, $R^2 = 0.09708$; IS/SPT: $F_{(1,2)} = 90.65$, $p= 0.0109$, $R^2 = 0.9784$). No additional correlations were found between circadian disruption and behavioral performance under LD conditions alone. In contrast, the combination of experimental LD conditions and prior alcohol access caused some correlations. Our data indicated a correlation between IS and SPT under LD24 (Regression analysis: $F_{(1,10)}= 11.97$, $p=0.0053$, $R^2=0.512$), RA and EPM under SHIFT (Regression analysis: $F_{(1,10)} = 5.297$, $p=0.0441$, $R^2=0.3463$), and IS and MBT under LD22 conditions (Regression analysis: $F_{(1,10)} = 6.188$, $p=0.0321$, $R^2 = 0.3823$) in animals with access to alcohol (Figure 11-B).

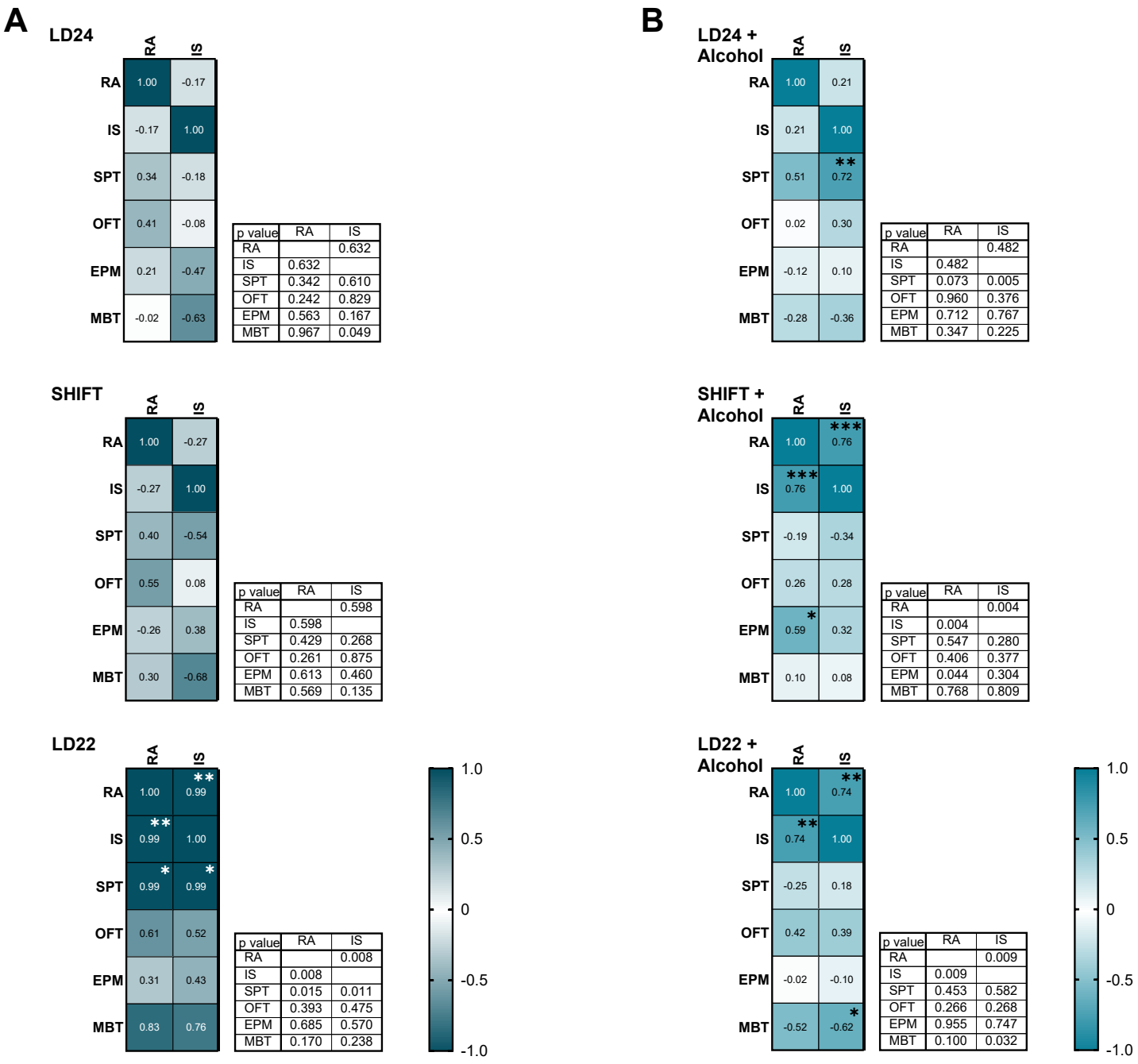


*Figure 11 Correlation between circadian disruption parameters and behavioral performance. (A) Behavioral performance of animals under LD24, SHIFT, and LD22 conditions and (B) in combination with alcohol abstinence a function of the relative amplitude during (RA) and interdaily stability (IS). The graph visually presents the correlation coefficient (Pearson r), and the statistical results, including p-values, are displayed alongside their corresponding graphs. Significant values are indicated in the graphs with *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ ±SEM; n = 4 - 10.*

2.4.3 Correlation between alcohol preference and behavioral performance

Further, we examined the correlation between alcohol consumption prior to and following a period of alcohol abstinence and behavioral performance (Figure 12). Consistent with our expectations, we found a significant correlation between alcohol preference before and after abstinence in animals housed under both standard and experimental LD conditions. A reduced alcohol preference during the first alcohol exposure period predicted a lower alcohol preference even after a prolonged alcohol abstinence period regardless of the exposed LD conditions (Regression analysis for LD24: $F_{(1,10)}= 7.158$, $p = 0.0233$, $R^2 = 0.4172$; SHIFT: $F_{(1,10)} = 27.30$, $p = 0.0004$, $R^2= 0.7319$; LD22: $F_{(1,10)} = 15.83$, $p = 0.0026$, $R^2 = 0.6128$).

Furthermore, a significant correlation emerged between the performance of animals in the OFT and their subsequent alcohol preference under LD22 conditions. Intriguingly, those animals that covered longer distances during the OFT exhibited a reduced preference for alcohol after an extended period of abstinence (Regression analysis: $F_{(1,10)}= 10.83$, $p = 0.0110$, $R^2 = 0.5751$). Additionally, these animals demonstrated a tendency toward lower initial alcohol preference. No other significant correlations were found between alcohol consumption and behavioral parameters.

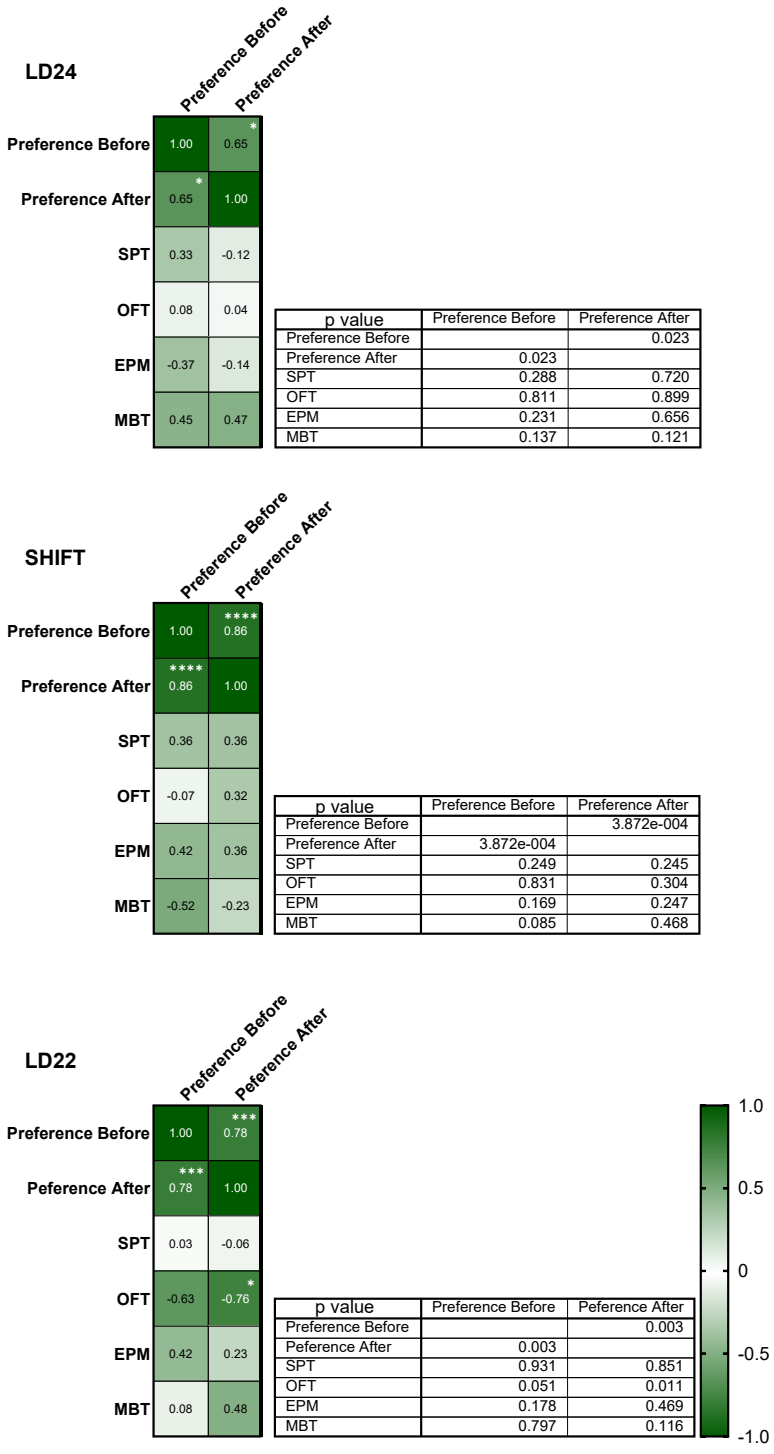


*Figure 12 Correlation between alcohol preference and behavioral performance. Alcohol preference, before and after abstinence (10 sessions respectively) related to the observed behavior in the SPT, OFT, EPM, and MBT. Statistical results are depicted next to the respective graph and contain the p values. Significant values are indicated in the graphs with **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ±SEM; $n = 10 - 12$.*

2.5 Discussion

Correlation and regression analyses are valuable tools for exploring potential relationships between different variables and identifying potentially important connections. Here we provided insight into the relationship between the degree of internal desynchronization and alcohol usage and further evaluated the effects of internal desynchronization and alcohol consumption on behavioral changes during alcohol abstinence (Figure 13).

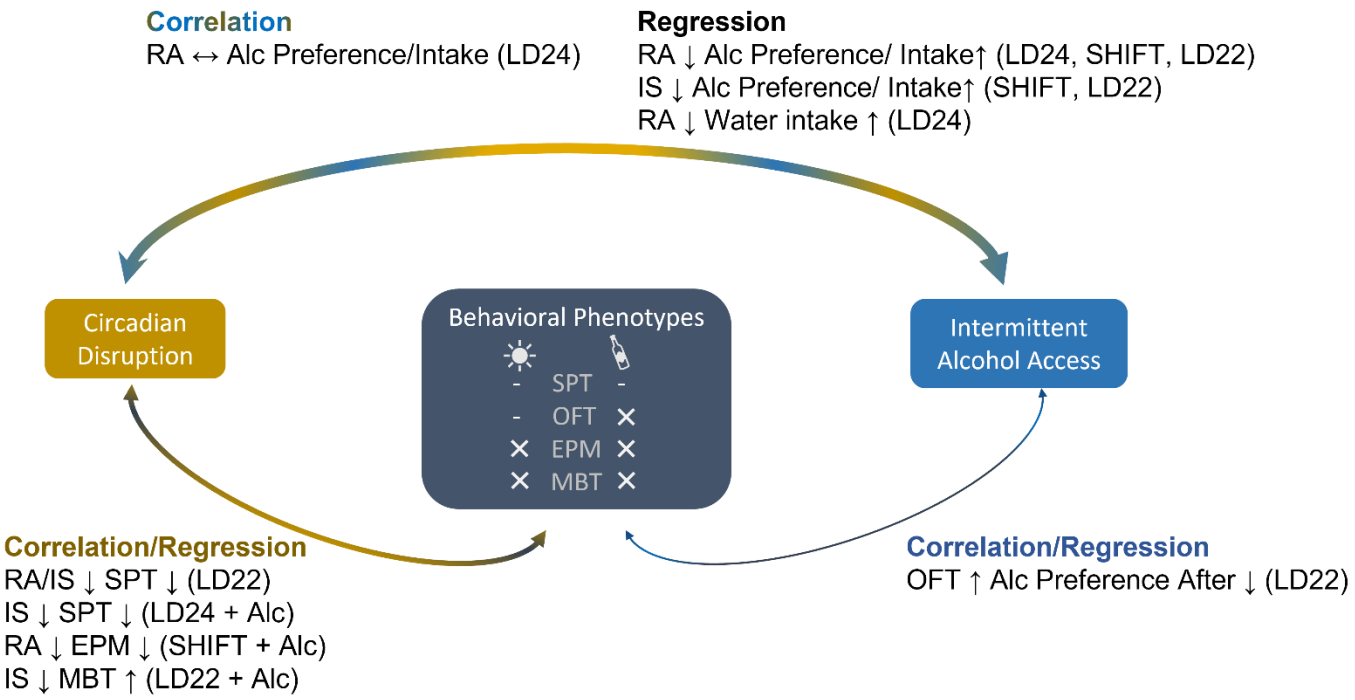


Figure 13 Relationship between circadian disruption and alcohol access on behavioral phenotypes.

2.5.1 Relationship between circadian disruption and alcohol consumption

Clinical studies indicated a significant relationship between shiftwork and an increased risk of alcohol consumption (Cheng et al., 2021; Richter et al., 2021). The odds of working at night, combined with poor sleep patterns, appear to be related to higher alcohol intake compared to day workers with poor sleep quality (Morikawa et al., 2013). Although genome-wide association studies in humans and experiments on clock-gene manipulation in animals indicated a link between clock gene malfunction and alcohol consumption, shiftwork conditions in human and animal studies found disparate results in alcohol intake behavior. Specifically in pre-clinical animal studies, individuals consume more or less alcohol depending on the used shiftwork and alcohol-drinking paradigms, animal strain, or sex. In comparison with genetic clock gene

manipulations, environmentally-induced circadian disruption is a persistent entrainment and re-entrainment challenge for the organism. Therefore, differences in the observed outcomes may be attributed to the species (human or animal), as well as other influences such as the experimental setup. In our experiment, we collected circadian output parameters and correlated them to alcohol intake behavior.

In the study, we observed a relationship between circadian disruption and alcohol consumption under standard LD24 conditions, but not under simulated shift-work conditions. However, our data may suggest a possible causal connection between the extent of circadian disruption and alcohol consumption in abnormal LD conditions, albeit relatively weak. Hence, lower rhythm stability and amplitude are indicative of increased alcohol intake. These results are interesting, as the data initially indicated no correlation between both variables, but regression analysis revealed that rhythm parameters may be a predictor for abnormal alcohol consumption. Correlation measures the strength of the relationship between two variables, while regression analysis examines the relationship between a dependent variable and one or more independent variables, considering the potential influence of other factors. While a simple examination of the data may not reveal this relationship, regression analysis allows for a more nuanced understanding of the interplay between the variables, highlighting their interconnected nature. Further, a correlation analysis may lack the power to detect a weak relationship, especially if the sample size is small. Hereby, the regression analysis can provide more robust estimates by considering the joint effects of multiple variables, even if the individual correlations are not significant. The regression analysis contained the repeated measures of alcohol intake (effect variable) over 10 exposure sessions versus the independent variable (cause), which was not included in the correlation analysis.

2.5.2 Correlation between circadian disruption and behavioral performance

Shiftwork has a significant impact on mood and behavior and studies associated shiftwork with an increased risk of depression and anxiety (Walker et al., 2020). Likewise, studies in animal models indicated the relationship between circadian disruption induced through clock gene manipulations or environmental manipulation and behavioral abnormalities (or mood-related phenotypes) (Ben-Hamo et al., 2016; Horsey et al., 2020; Landgraf et al., 2016). Previous studies using correlation analysis indicated a relationship between circadian disruption and behavior. Ben-Hamo and colleagues reported a negative relation between the amplitude of locomotor

activity rhythms and depressive-like behavior in male rats (Ben-Hamo et al. 2016), and Banks and colleagues demonstrated that circadian disruption (using IS as a measure) is positively correlated with the latency to enter the center of the open field in female mice but not in male mice (Banks et al., 2022). Notably, females have a stronger daily rhythm and higher voluntary activity under standard LD conditions, and a higher RA, lower IV, and IS under disruptive LD conditions compared to male mice, and sex differences in the correlation between circadian disruption and behavioral performance are observed (Banks et al., 2022).

We observed changes in the behavior during alcohol abstinence (refer to Chapter 1), with some associations to the used LD paradigm and/or intermittent alcohol access. Following, we correlated the downstream behavior with the degree of circadian disruption. Overall, we did not identify a strong correlation between circadian disruption and behavioral observations.

2.5.3 Correlation between alcohol preference and behavioral performance

Alcohol consumption has been strongly linked to affective disorders in humans (Boden & Fergusson, 2011), as well as mood-related changes in animals (Amodeo et al., 2018; Conte et al., 2022). Mood disorders often coexist with alcohol dependence (Lynskey, 1998). Furthermore, repeated exposure to alcohol has been shown to have significant neural and cognitive consequences based on findings from both human and animal studies (Spear, 2018).

Studies investigating the effects of adolescent alcohol exposure in rodents have traditionally encompassed assessments of cognitive functioning as well as various behaviors such as risk-taking, impulsivity, anxiety, and social interactions (Cacace et al., 2012; Salimov, 1999). In our study, we used the collected dataset and conducted a correlation analysis to examine the relationship between individual alcohol intake behavior and corresponding behavioral performance. Surprisingly, we observed only one significant association between observed behavior and alcohol consumption. This link was found between alcohol preference after abstinence and the distance traveled during the Open Field Test (OFT) under LD22 conditions. This finding is consistent with what we previously observed in Chapter 1, where alcohol access influenced the animals' performance in the OFT. As a result, it appears that the intermittent alcohol access paradigm employed in our study did not show a clear link to the observed behavior during alcohol abstinence in female rats.

2.5.4 Limitations

In this study, we investigated the effects of circadian disruption and alcohol on behavioral outcomes. The most notable correlation was observed between circadian disruption and behavior, with no strong correlation detected between circadian disruption and alcohol consumption, or between alcohol consumption and behavior. A potential causal relationship between alcohol consumption and disrupted circadian rhythms was found to be weak.

It is possible that the alcohol-drinking paradigm we used may not be sufficient to induce severe behavioral consequences in female rats. Although the intermittent alcohol access paradigm leads to an escalation of alcohol consumption over time in male and female rodents, we did not find any differences based on the light-dark schedule of the animals' housing. It may be interesting to explore alternative schedules, such as drinking in the dark, which simulates binge-drinking behavior (Thiele & Navarro, 2014) and has been shown to induce changes in gene expression (Marballi et al., 2016).

Another important aspect to consider is the use of circadian parameters, specifically RA and IS, as behavioral indicators of the circadian system. These non-parametric variables of rhythmicity are calculated based on locomotor activity. Although locomotor activity is related to mood-associated behavior in rats under altered light-dark conditions (Anyan et al, 2017) other factors, such as hormones like estrogen, may also influence locomotor activity (Espinosa & Curtis, 2018). Moreover, individual differences among animals can further complicate the correlation analysis when including behavioral variables (Anyan et al., 2017). Additionally, the dataset consisted of female animals, and sex differences in behavioral outcomes have been reported. Further studies should investigate the role of circadian disruption and alcohol consumption on behavior in male rats and compare the results to those obtained from female animals.

While correlation analysis is a useful tool, it can be challenging to interpret because it may be influenced by confounding variables that can obscure or exaggerate relationships. Confounding variables are factors that are not directly related to the variables being studied but can impact the results. For instance, individual preferences, or individual characteristics, or external factors can influence outcomes, such as variation in the chronotype or entrainment as reported in squirrels and mice (Pfeffer et al., 2015; Refinetti et al., 2019). Like in humans, differences in chronotypes

and entrainment have implications for many biobehavioral processes, such as cognitive performance, mood, and impulsivity.

It is important to note that correlation analysis does not establish causation, and additional research is often necessary to determine underlying mechanisms and establish causal relationships. Despite these limitations, correlation analysis helps identify potential relationships between variables and generates hypotheses for further investigation. It also highlights areas where more detailed analyses, such as regression or experimental studies, may be required to establish causality or identify confounding variables.

Additionally, future studies should consider incorporating larger sample sizes, diverse populations, and longitudinal designs to strengthen the validity and generalizability of the findings. Furthermore, investigating the potential role of genetic factors, environmental influences, and individual differences in the relationship between circadian disruption, alcohol consumption, and affective behavior would provide valuable insights. Utilizing advanced techniques such as neuroimaging or molecular analyses could help to elucidate the specific neurophysiological pathways involved. Hence, a multidisciplinary approach combining behavioral, physiological, and genetic perspectives will be essential for advancing our understanding of these complex interactions and informing potential interventions and treatments for individuals affected by chronodisruption, alcohol misuse behavior, and associated affective disorders.

2.6 Conclusion

In summary, the analysis conducted in this chapter aimed to relate the degree of circadian disruption and alcohol consumption to behavioral outcomes. We found a correlation between circadian disruption and behavior, indicating that disruptions in the internal clock may influence behavioral patterns. However, we did not observe a strong correlation between circadian disruption and alcohol intake, nor between alcohol intake and behavior. At an individual level, some behaviors may be influenced by past episodes of circadian disruption, while others are not affected. It is worth noting that the relationship between environmentally-induced internal desynchronization, alcohol intake, and downstream behavioral performance may be more complex and multifaceted. Individual differences, genetic factors, and environmental influences can also play a role in the development and manifestation of these conditions. Hence, the

relationship between the manipulation of variables and the assessment of downstream behavior may be more complex, and caution should be taken in terms of the validity and discussion of these behavioral datasets.

Chapter 3: The effects of dysregulated circadian rhythms and alcohol consumption on the expression of circadian and cell-signaling genes in brain areas involved in the reward system in female rats

Christiane Meyer, Konrad Schoettner, Shimon Amir

Manuscript draft that is yet to be submitted to an academic journal (in preparation).

Author Contributions

CM and SA conceived and designed the experiments. CM conducted the experiments and was assisted by KS. CM analyzed and interpreted the data with input from KS and SA. CM did the data visualization. CM wrote the manuscript and revised by SA and KS. SA supervised the project.

3.1 Abstract

Circadian dysfunction is associated with altered alcohol intake in humans and animals. The impact of environmental LD changes on gene functioning in brain areas related to alcohol misuse has been a topic of interest. Brain region-specific changes in mRNA expression of key clock genes have been observed under circadian disruption and alcohol use. However, the combined effect of circadian disruption and alcohol consumption on gene expression in the brain is poorly understood. Strikingly, most previous research was carried out on male subjects, with unclear implications for the female sex.

To address this gap in knowledge, adult female rats housed under an 11h/11h light-dark schedule (LD22) were given access to intermittent alcohol (IA20%) to assess the gene expression in brain areas implicated in alcohol consumption and reward, the prefrontal cortex (PFC), nucleus accumbens (NAc) and dorsal striatum (DS). We measured mRNA expression of core clock genes (*Bmal1*, *Clock*, *Per2*), sex hormone receptors (*ERβ*, *PR*), glutamate receptors (*mGluR5*, *GluN2B*), calcium-activated channel (*Kcnn2*), and pro-inflammatory marker (*TNFα*) at two-time points relative to the locomotor activity cycle. Additionally, we determined changes in locomotor activity and alcohol-drinking behavior.

Housing under LD22 did not affect alcohol intake but significantly disrupted circadian rhythms and reduced locomotor activity. Significant changes were evident in *Bmal1*, *ERβ*, and *Kcnn2*, whereas *Per2* and *PR* were affected by intermittent access to alcohol. Only *TNFα* was affected by both the aberrant LD cycle and alcohol. Collectively, these results indicate that disruption of circadian rhythms and/or intermittent alcohol exposure initiate changes in gene expression which may have implications for the progression of alcohol intake in females.

Keywords: Alcohol, females, gene expression, clock genes, glutamate receptors, neuroinflammation, hormone receptors.

3.2 Introduction

Alcohol use disorder is a global public health concern contributing to millions of premature deaths each year (WHO, 2018). Various factors influence alcohol consumption, including circadian rhythm disruption. Research has shown that working night shifts or rotating schedules can increase the risk of alcohol abuse (Richter et al., 2021), while genetic variations in clock genes are linked to alcohol dependence and increased alcohol intake (Kovanen et al., 2010). Long-term alcohol consumption also interferes with the circadian system, which can further promote alcohol intake (Spanagel, Rosenwasser, et al., 2005). To understand the interplay between circadian rhythm disruption and alcohol consumption and their impact on health it is crucial to address their sole and combined effect on the organism.

The circadian system regulates daily (circadian) biological rhythms in metabolism, physiology, and behavior through a network of biological clocks present in every cell of the body. These clocks are based on an interplay between positive regulators such as the Brain and muscle arnt-like 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK), and negative regulators such as Period (Per) and Cryptochrome (Cry), which together create a 24-hour feedback loop. Disruptions to this circadian system, as experienced under shift work conditions, not only affect the core circadian feedback loop but also impact other genes, so-called clock-controlled genes, which play essential roles in cellular functions such as metabolism and cell signaling (Bozek et al., 2009).

In research studies, both environmental and genetic manipulations have been used to induce circadian disruption to investigate its effects on alcohol intake behavior. Experimental LD conditions that aimed to induce chronic misalignment of circadian rhythms, showed varied effects on alcohol intake in animal models. Studies reported an increase in alcohol intake in male Sprague-Dawley and female HAD-1 rats (Clark et al., 2007; Gauvin et al., 1997), whereas other studies observed no changes or reduced alcohol intake in female and male Lewis, Fisher, and Wistar rats (Clark et al., 2007; Meyer et al., 2022; Rosenwasser et al., 2010). In contrast, studies targeting specific clock genes revealed a clear relationship between the clock gene system and alcohol consumption. The knockout of *Per2* and *Clock* has been shown to increase alcohol consumption in mice (Ozburn et al., 2013; Spanagel, Pendyala, et al., 2005), while deficiencies

in genes like *Rev-erbα* and *Cry1/2* have been associated with reduced alcohol preference (Al-Sabagh et al., 2022; Hühne et al., 2022).

Furthermore, alcohol consumption has been found to affect the circadian system at the molecular and behavioral levels. Alcohol intake alters circadian behavioral outputs such as the free-running period, locomotor activity, and light entrainment (Brager et al., 2010; Logan et al., 2010; Rosenwasser et al., 2005). At the molecular level, the expression pattern of *Per2* in the suprachiasmatic nucleus (SCN) and the arcuate nucleus can be altered by alcohol consumption (Chen et al., 2004). However, other studies suggest that chronic alcohol consumption disrupts the molecular clock in the liver without affecting the expression of clock genes in the SCN (Filiano et al., 2013). Taken together, these studies indicate that alcohol consumption can interfere with the functioning of the circadian clock, but our understanding of how alcohol impacts clock gene function in brain regions beyond the SCN remains limited.

Lastly, sex differences in alcohol consumption have been reported in both human and animal models, and there is emerging evidence that circadian clocks or their components contribute to these differences (de Zavalia et al., 2022). The circadian timing system exhibits sex differences with molecular and behavioral implications for both sexes (Yan & Silver, 2016), which may contribute to disparities in alcohol consumption patterns. Although sex differences in alcohol intake behavior in animals with disrupted circadian rhythms have been reported (Rizk et al., 2022; Rosenwasser et al., 2014), most studies were still carried out with males. Thus, it remains elusive how circadian disruption affects alcohol consumption in females, and whether this could be associated with changes in clock gene expression in brain areas that are linked to alcohol-drinking behavior and vice versa.

To address this gap in knowledge, we tested the distinct and combined effects of aberrant light exposure and intermittent alcohol access on gene expression in the brains of adult female rats. Whereas chronodisruption did not affect drinking behavior, we found that the combination of altered LD and alcohol consumption modified the expression of clock genes and genes associated with proinflammatory responses. Our results contribute to the understanding of the molecular consequences of circadian disruption and/or alcohol use in female rats and may help in the development of targeted interventions and recommendations to treat alcohol abuse in females.

3.3 Materials and methods

3.3.1 Ethics statement

All experimental protocols were approved by the Concordia University Animal Care Committee (certificate number: 30000256).

3.3.2 Animals and light-dark conditions

Adult female Wistar rats were purchased from Charles River (St. Constant, QC, Canada). Animals were singly housed in clear plexiglass cages (9.5"x8"x16") equipped with running wheels in light (standardized illuminance of~200–300 lux during the light period, 0 lux during the dark period) and temperature- and humidity-controlled rooms (temperature: 21±1◦C, relative humidity: 65±5%). Wheel-running activity of each animal was continuously recorded with VitalView system (Mini-Mitter, Starr Life Sciences Corp., Oakmont, USA). Food and water were available *ad libitum*. Only animals with a regular estrous cycle were included in the study.

Following a 3-week habituation to the housing and experimental setting, half of the animals were placed under LD22 (11:11 h light-dark, n= 6), and the other half remained under standard LD24 (12:12 h light-dark, n= 6) conditions. The LD22 light paradigm has been shown to disrupt circadian rhythms and to affect temperature, sleep, corticosterone secretion, and mood (Ben-Hamo et al., 2016; Cambras et al., 2007; De La Iglesia et al., 2004; Wotus et al., 2013).

3.3.3 Intermittent alcohol drinking paradigm

Female rats kept under LD24 (n= 6) or LD22 (n= 6) were given access to 17 daily intermittent alcohol (20% v/v, in tap water) exposure sessions (IAE20%) as previously described (Wise, 1973). On alcohol days, animals were given access to one bottle of 20% alcohol solution and one bottle of water. After 24 hours, the bottles were replaced with two water bottles that were available for the next 24 hours. This pattern was repeated each Monday, Wednesday, and Friday. Over the weekend animals had access to water for 48 hours. To control for side preferences, the position of the alcohol and water bottles was switched for each alcohol exposure session. Alcohol intake (g/kg body weight/day), and alcohol preference (g of alcohol solution consumed/ g of total fluid intake) were measured and calculated at the end of each daily alcohol session.

3.3.4 Gene expression

Gene expression was assessed in tissue punches collected from the prefrontal cortex (PFC), nucleus accumbens (NAc), and dorsal striatum (DS). Animals were killed 24h to 72h after the last alcohol exposure at ZT2 (n= 3) and ZT14 (n= 3) (ZT- Zeitgeber time; ZT0 represents the time of light onset). In animals kept under LD22, ZT2 refers to two hours after activity offset and ZT14 to two hours after activity onset. Animals kept under LD24 were killed at the stage of proestrus. Animals exposed to LD22 did not show a regular estrous cycle (Meyer et al., 2022). The estrous cycle stage was visually determined by the locomotor activity pattern over four days, as previously described (Wollnik & Turek, 1988). After decapitation, brains were flash-frozen and stored at -80°C until further processing. 200µm thick coronal sections were obtained using a cryostat (Microm HM505 E, Microm International GmbH, Walldorf, Germany), and tissue punches (2 mm in diameter) were collected from each hemisphere following the rat brain atlas (Paxinos & Watson, 2006).

Total RNA isolation was conducted following a standard Trizol extraction protocol according to the manufacturer's instructions (Invitrogen™, Life Technologies Corporation, Carlsbad, CA, United States) and total RNA yield was measured using spectrophotometry (Nanodrop 2000; Thermo Scientific™, Wilmington, DE, United States). Next, the RNA integrity was determined by the "bleach gel" method (Aranda et al., 2012). cDNA was synthesized from 1µg of RNA using the iScriptTMReverse Transcription Supermix following the manufacturer's instructions (Biorad, Hercules, CA, United States). Besides the standard cDNA samples, a no reverse transcriptase (no-RT) control was prepared.

Realtime PCR was performed with SYBR®Green Supermix SYPR (Biorad, Hercules, CA, United States) and amplification was done using the CFX96TM Real-Time PCR system (Biorad, Hercules, CA, United States). Quantification of target genes was calculated by using the delta-delta Ct (ΔΔCt) method (Pfaffl, 2001) by the CFX Maestro qPCR Software (Biorad, Hercules, CA, United States). The Ct values were normalized to those of β-actin and Gapdh. The primers for the used genes are shown in Table 5.

Target gene	Forward Primer	Reverse Primer
Gapdh	CTTGTGCAGTGCCAGCCTC	GGTAACCAGGCGTCCGATAC
β-Actin	CGCGAGTACAACCTTCTTGC	CGTCATCCATGGCGAACTGG
Bmal1	TGGACTGCAACCGCAAGAG	CCTTCCATGAGGGTCATCTT
Per2	GACGGGTCGAGCAAAGGA	GGGAAAAGTCCACATATCCA
Clock	GCGAGAACTTGGCATTGAG	GGAGGAGGCAGAAGGAGTTG
ERβ	GCTGGGCCAAGAAAATCCCT	CCCCTCATCCCTGTCCAGAA
PR	CTCGCTGTGCCTTACCATGT	TTGACTCCTCAGGCCTTCCA
mGluR5	TGAGTTAAGTCACCAGGTGC	TTCTGCTGGCTGCCACTAC
mGluN2B	AGATGCCAGCTGAGCCTTG	AGGATGAAGCGTGACTTGGA
TNFα	GATCGGTCCCAACAAGGAGG	TTTGCTACGACGTGGGCTAC
Kcnn2	ACTTCCTTGGAGCAATGTGG	GTGCAACCTGCACCCATTAT

Table 5 Primer list for quantitative real-time PCR.

3.3.5 Data analysis and statistics

Data from locomotor and circadian measurements as well as gene expression were analyzed using Prism 9 (GraphPad Software, San Diego, CA, United States). Results were expressed as mean ± standard error of the mean (SEM) and were examined for normality and homogeneity of variance. The significance level was set at $p< 0.05$.

Circadian rhythmicity was analyzed by continuous registration of locomotor activity using running wheels. Summed wheel revolutions were recorded over 10-minute periods using the VitalView system (Mini-Mitter, Starr Life Sciences Corp., Oakmont, USA). Wheel running activities were visualized as double-plotted actograms using ClockLab 6 (Actimetrics Software, Evanston, United States). The circadian rhythm parameter, Relative Amplitude (RA), Intradaily Variability (IV), and Interdaily Stability (IS) were calculated using ClockLab 6 (Actimetrics Software, Evanston, United States) over the last ten days of the experiment. Differences between the treatment groups were calculated by a one-way ANOVA, followed by Tukey's multiple comparisons tests.

Locomotor activity counts were determined over eight days during habituation and eight days at the end of the experiment. As locomotor activity varies in female rodents depending on the stage of the estrous cycle, we assessed the activity counts over eight days as a multiplicand of a four-day estrous cycle. Changes in locomotor activity during treatment over baseline activity were calculated in percentage.

Alcohol consumption and alcohol preference over water were assessed by a Two-way repeated measures ANOVA (LD and alcohol session effect). A three-way ANOVA (LD, fluid condition, and ZT effect) followed by Šídák's multiple comparisons test was used to assess results from the gene expression analysis.

3.4 Results

3.4.1 Locomotor activity and circadian rhythm parameter

We first assessed the locomotor activity of rats kept under LD24, LD22, and in combination with the IA20% paradigm. The visual inspection of the actograms showed that the LD condition affects locomotor activity, but not alcohol (Figure 14). As expected, the circadian parameters IS and the RA differed between LD groups (Figure 15; One-way ANOVA, IS: $F_{(3,20)}$= 11.78, p=0.0001; RA: $F_{(3,20)}$= 10.13 p= 0.0003). However, the IV did not vary between LD24 and LD22 (IV: $F_{(3,20)}$= 0.6710, p= 0.5798). We did not observe an added effect of alcohol on the assessed circadian parameters.

Further analysis of the home cage activity counts revealed significant suppression of locomotor activity under the LD22 condition, but no effect of alcohol (Figure 16; Two-way ANOVA: Fluid Condition p= 0.4676; LD Condition p= 0.0041, Interaction p= 0.7811).

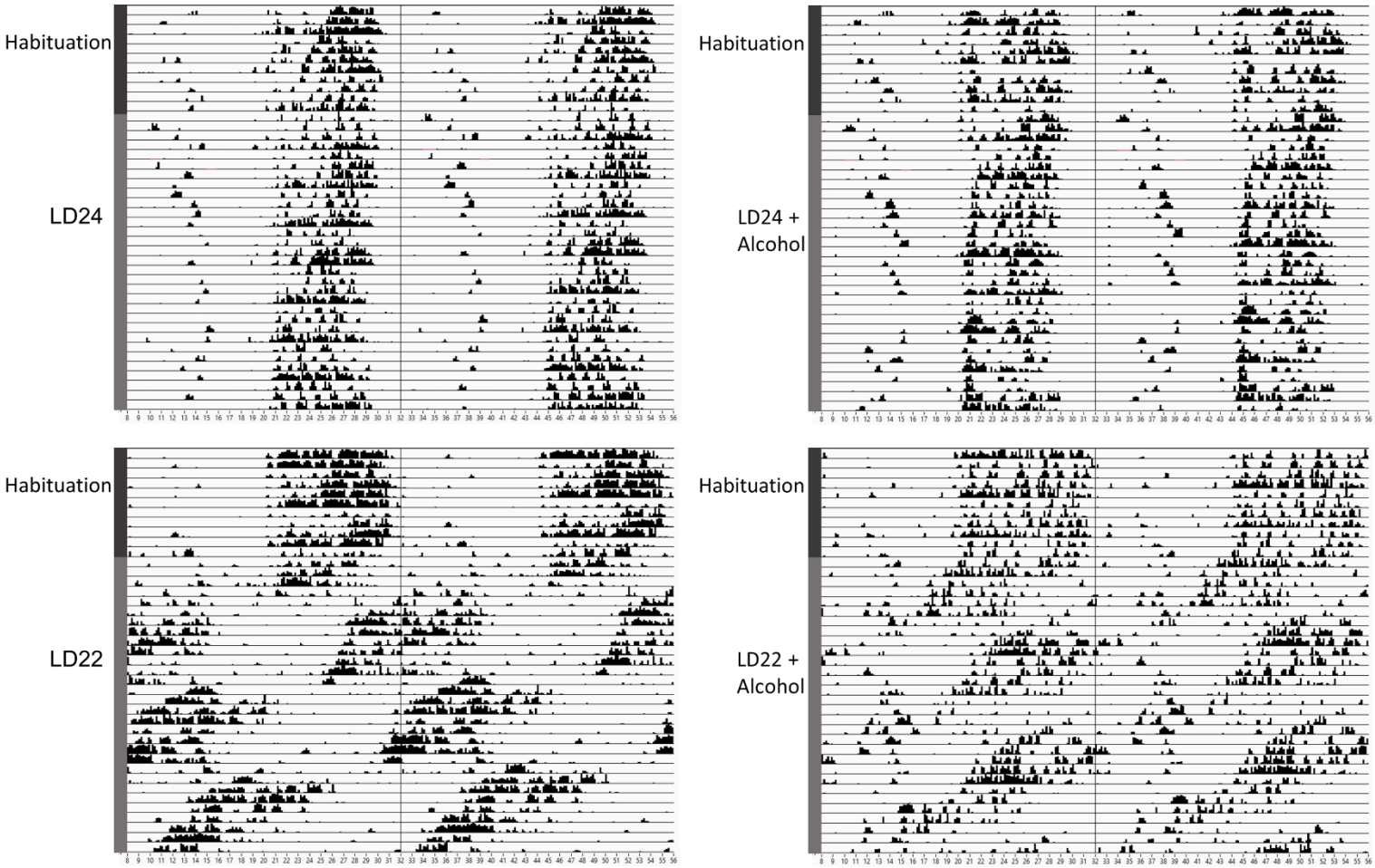


Figure 14 Representative double-plotted actograms depicting the locomotor activity of animals kept under LD24 and LD22 conditions with access to water or intermittent alcohol over 40 days. The time of day is double plotted along the x-axis, and successive days are arranged from top to bottom along the y-axis.

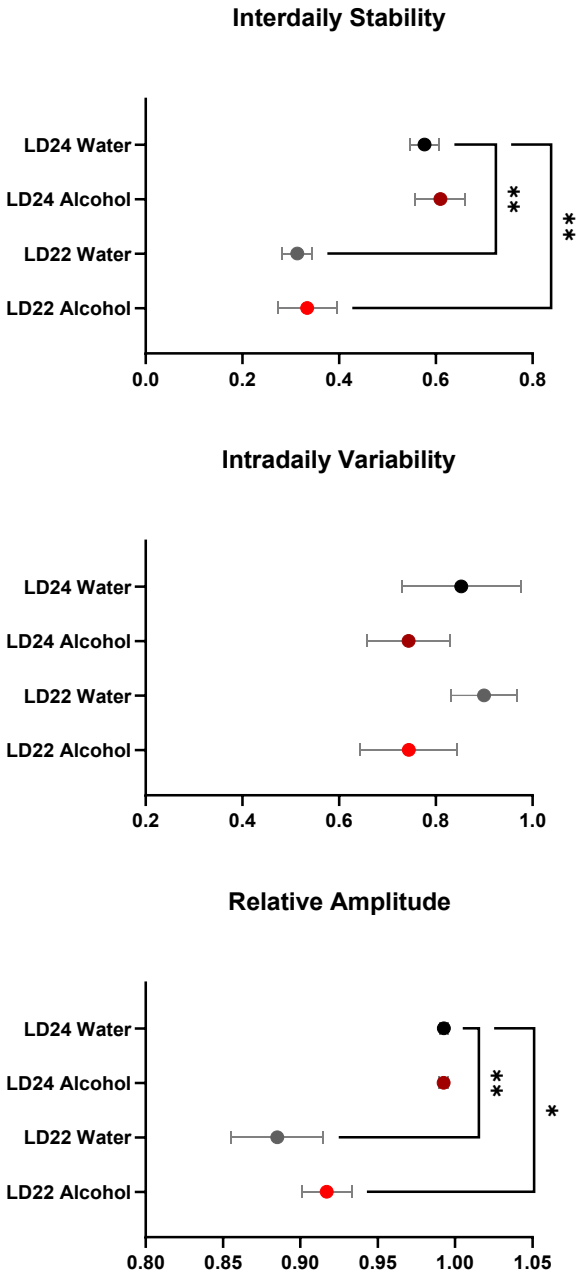


*Figure 15 Change of circadian parameters during the last 10 days of the experiment. Interdaily Stability and Relative Amplitude were significantly affected by the LD condition. Intradaily Variability was not affected. One-way ANOVA, followed by Tukey's multiple comparisons test * p< 0.05, ** p< 0.01. Data are mean ± SEM, n=6 animals per LD, and fluid condition.*

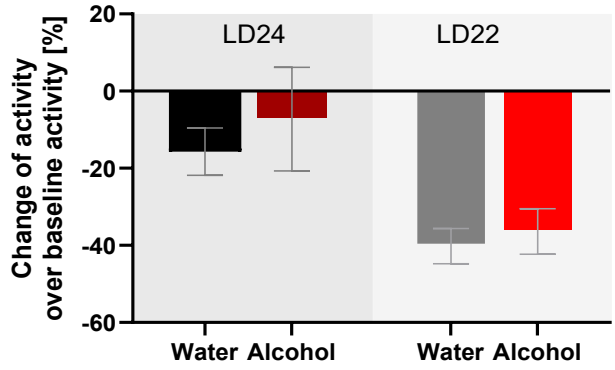


Figure 16 Change of home cage activity during treatment over baseline activity. The sum of activity counts of 10-minute intervals over eight days. Data are mean ± SEM, n= 6 animals per LD, and fluid condition.

3.4.2 Alcohol drinking behavior

All rats significantly increased alcohol intake and preference over time, regardless of LD condition (Figure 17; Alcohol consumption: Session $F_{(3.975, 39.75)}$= 6.383 p= 0.0005; Alcohol preference: Session $F_{(4.898, 48.98)}$= 4.566 p= 0.0018). However, no differences in intake and preference were observed between the LD groups (Two-way ANOVA; LD $F_{(1, 10)}$= 1.160, p= 0.3068) and alcohol preference (Two-way ANOVA: LD $F_{(1, 10)}$= 0.3119, p= 0.5888).

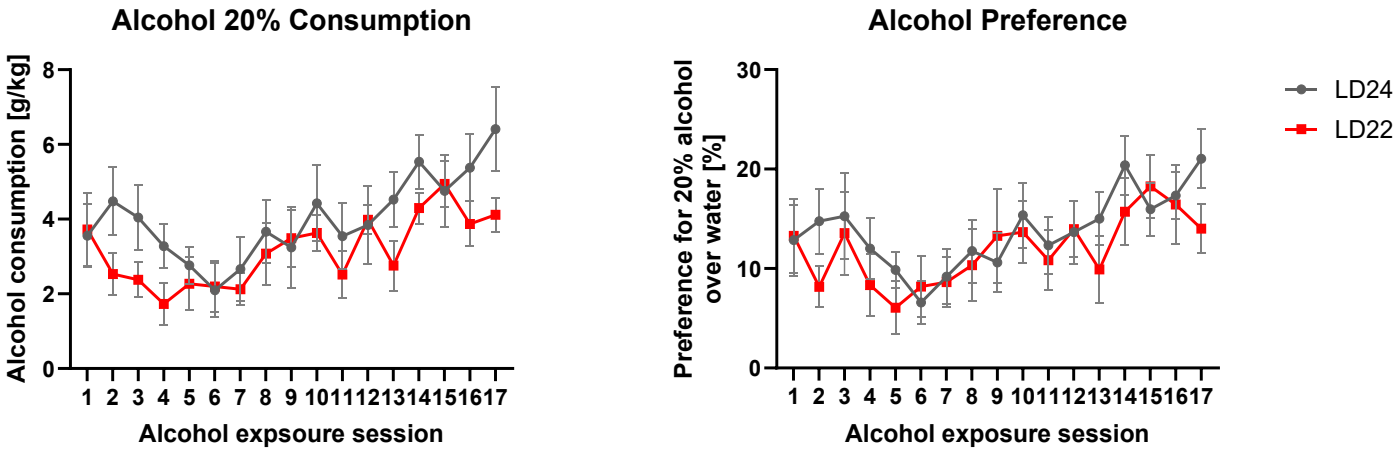


Figure 17 Alcohol consumption and preference of female rats. Intermittent alcohol drinking behavior is not affected by the LD condition. Animals had access to two bottles containing either water or alcohol (20%, v/v) every other day and were allowed 24-hour access. On non-alcohol days, both bottles contained water. Data are mean ± SEM, n= 6 animals per LD condition.

3.4.3 Gene expression

Using real-time polymerase chain reaction (PCR), we examined the gene expression changes of three key clock genes (*Bmal1*, *Per2*, and *Clock*) in the PFC, NAc, and DS of rats exposed to different LD and fluid conditions (Figure 18, Table 6). Visual inspection of the investigated clock genes clearly showed a two-timepoint oscillation under standard LD24 conditions in *Bmal1* and *Per2*, but not in *Clock* (Figure 18). The three-way ANOVA of *Bmal1* revealed an effect of ZT on the *Bmal1* expression in all three brain regions and an interaction of ZT with LD condition in the NAc and DS, but no effect of LD and alcohol (Table 6). Šídák's multiple comparisons test showed a significant ZT dependence in the NAC and DS between ZT2 and ZT14 under standard LD24 that vanished under LD22 conditions. In contrast, *Per2* is significantly affected by the change in the fluid conditions and ZT in all three brain regions and an interaction between ZT and fluid or LD conditions was found in the NAc (Table 6). The post hoc analysis indicated differences between ZT2 and ZT14 under all conditions, except in the PFC of animals with access to alcohol. Interestingly, the expression of *Clock* was not affected by LD, alcohol, or the examined time points (ZT2, ZT14) as confirmed in the three-way ANOVA (Table 6).

We further examined the mRNA expression of the hormone receptors *ERβ* and *PR* (Figure 19). Although both hormone receptors were not markedly affected by the experimental conditions, the three-way ANOVA revealed changes in the expression of *ERβ* depending on the ZT in the PFC and LD conditions in the PFC and DS. The expression of *PR* mRNA was only affected by the fluid condition in the PFC (Table 6).

Following, we examined genes related to glutamate signaling, namely the glutamate receptors *mGluR5* and *GluN2B*, and the small conductance calcium-activated potassium channel protein 2 (*Kcnn2*) (Figure 20). We observed changes in the mRNA expression of *mGluR5* in the interaction of fluid condition and LD or ZT in the NAc and DS, but not for the *GluN2B* receptor (Table 6). The *Kcnn2* mRNA expression was altered in the PFC depending on the LD and in the NAc depending on the ZT condition.

Lastly, we examined the expression of the pro-inflammatory marker *TNFα* (Figure 21). Our data show that LD22 and alcohol access significantly affected the mRNA expression of *TNFα* independently from each other. The change in LD condition altered the mRNA expression of

TNFα in all three brain regions, whereas fluid conditions affected the mRNA levels in the NAc and DS (Table 6). The post hoc test did not reveal significant changes between each condition.

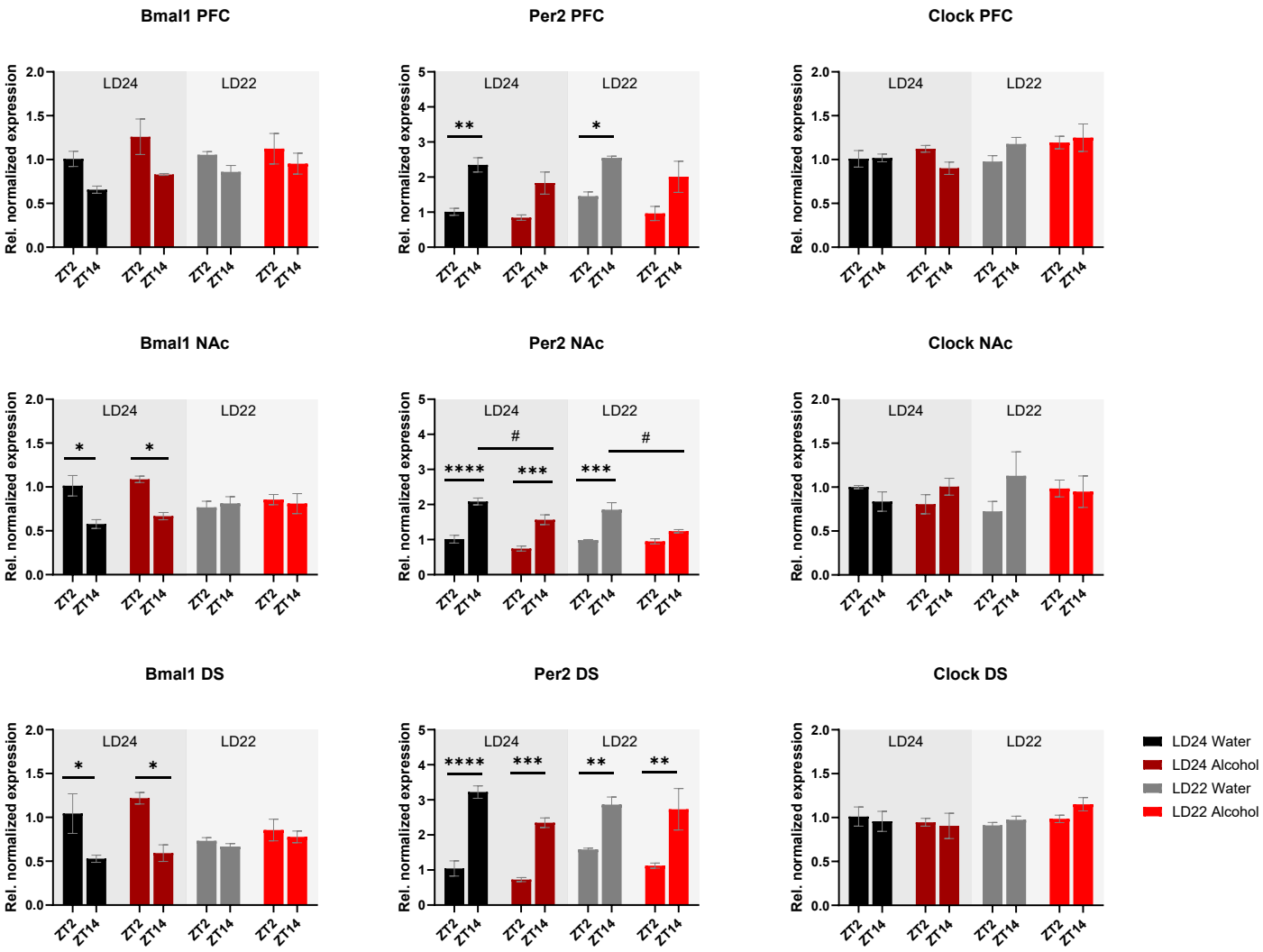


Figure 18 Gene expression of Bmal1, Per2, and Clock mRNA in the prefrontal cortex (PFC), nucleus accumbens (NAc), and dorsal striatum (DS) at ZT2 and ZT14. Animals were exposed to different LD conditions (LD24 or LD22) and had access to water or intermittent alcohol access. The gene expression of the clock genes Bmal1 and Per2 are affected by the LD condition or fluid condition in contrast to Clock (for details see the result section and Table 6). Three-way ANOVA, Šídák's multiple comparisons test: ∗∗∗∗ p< 0.0001, ∗∗∗ p< 0.001, ∗∗ p< 0.01, ∗ p< 0.05. Data are mean ± SEM, n= 3 animals per LD condition and fluid condition.

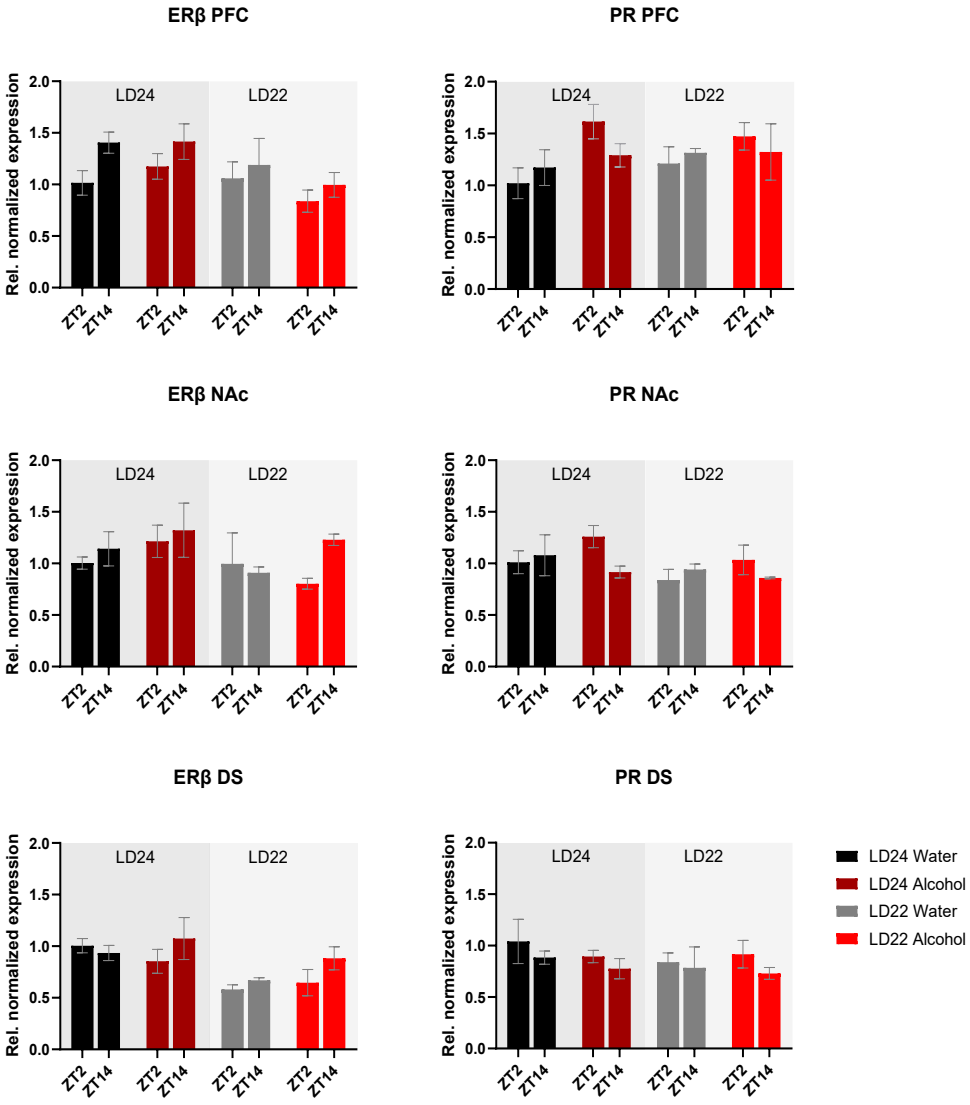


Figure 19 Gene expression of ERβ and PR mRNA in the PFC, NAc, and DS at ZT2 and ZT14. Animals were exposed to different LD conditions (LD24 or LD22) and had access to water or intermittent alcohol. The gene expression of ERβ and PR are only minimally affected by the LD condition or fluid condition (for details see the result section and Table 6). Data are mean ± SEM, n= 3 animals per LD condition and fluid condition.

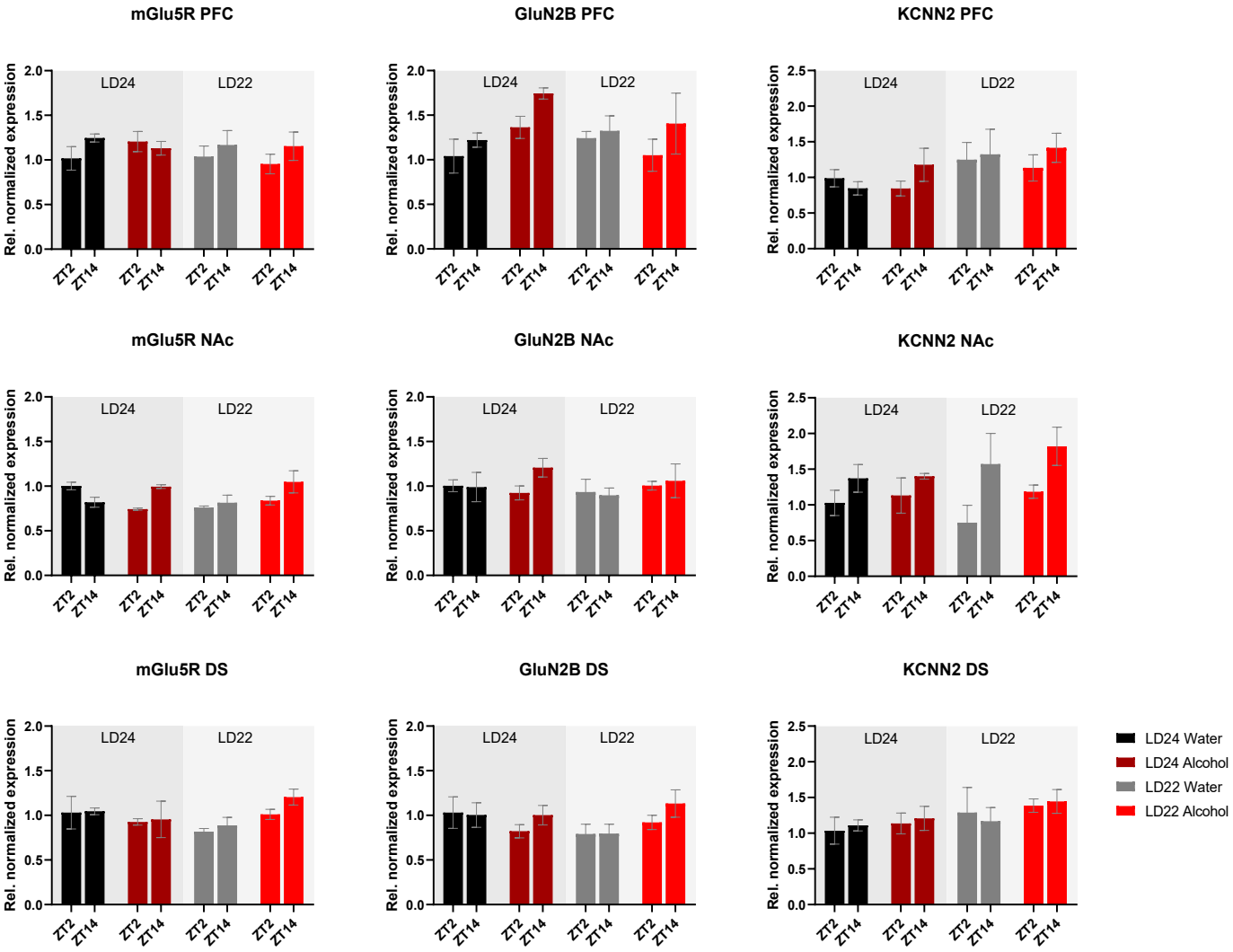


Figure 20 Gene expression of Glutamate receptors (mGluR5, GluN2B) and small conductance calcium-activated potassium channel protein 2 (Kcnn2) mRNA in the PFC, NAc, and DS at ZT2 and ZT14. Animals were exposed to different LD conditions (LD24 or LD22) and had access to water or intermittent alcohol exposure. The expression of mGluR5 was affected by the interaction of LD and fluid condition, whereas no changes in the GluN2B receptor mRNA were observed. The mRNA levels of Kcnn2 were slightly increased under LD22 conditions in the PFC and NAc (for details see the result section and Table 6). Data are mean ± SEM, n= 3 animals per LD condition and fluid condition.

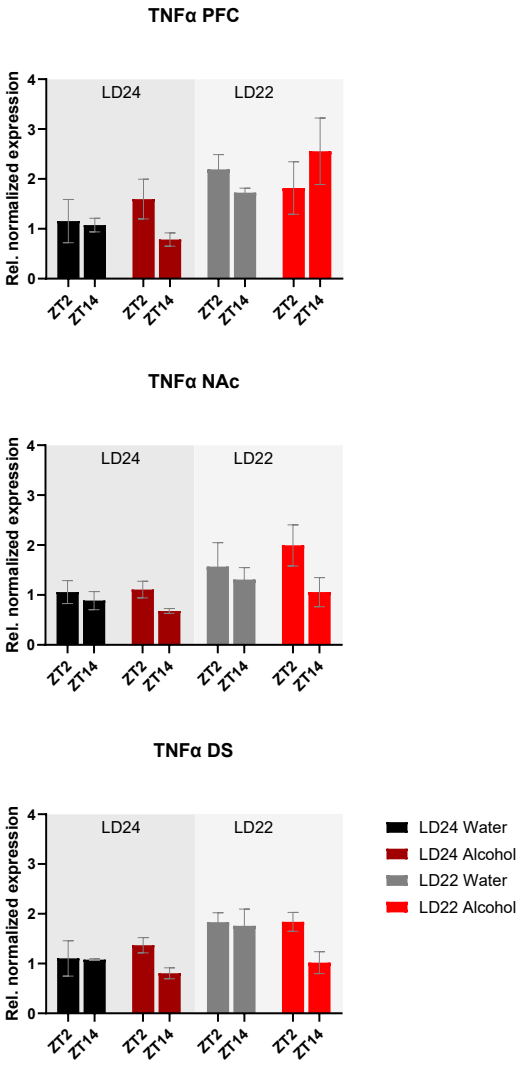


Figure 21 Gene expression of TNFα mRNA in the PFC, NAc, and DS at ZT2 and ZT14. Animals were exposed to different LD conditions (LD24 or LD22) and had access to water or intermittent alcohol exposure. The expression of TNFα was affected by LD22 and fluid condition (for details see the result section and Table 6). Data are mean ± SEM, n= 3 animals per LD condition and fluid condition.

Target	Brain Region	Three-way ANOVA			
		LD Condition	**Fluid Condition**	**ZT**	**Interactions**
Bmal1	PFC	n.s. (p= 0.4655)	n.s. (p= 0.0825)	**p= 0.0024**	ZT x LD Condition: n.s. (p= 0.2100) ZT x Fluid Condition: n.s. (p= 0.8646) LD Condition x Fluid Condition: n.s. (p= 0.4204) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7512)
	NAc	n.s. (p= 0.6497)	n.s. (p= 0.2625)	**p= 0.0011**	ZT x LD Condition **p= 0.0011** ZT x Fluid Condition: n.s. (p= 0.7389) LD Condition x Fluid Condition: n.s. (p= 0.7260) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6195)
	DS	n.s. (p= 0.2562)	n.s. (p= 0.1312)	**p= 0.0005**	ZT x LD Condition **p= 0.0041** ZT x Fluid Condition: n.s. (p= 0.6825) LD Condition x Fluid Condition: n.s. (p= 0.9883) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7479)
Per2	PFC	n.s. (p= 0.1609)	**p= 0.0167**	**p< 0.0001**	ZT x LD Condition: n.s. (p= 0.7798) ZT x Fluid Condition: n.s. (p= 0.5426) LD Condition x Fluid Condition: n.s. (p= 0.5904) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6400)
	NAc	n.s. (p= 0.2296)	**p= 0.0003**	**p< 0.0001**	ZT x LD Condition **p= 0.0284** ZT x Fluid Condition **p= 0.0162** LD Condition x Fluid Condition: n.s. (p= 0.6452) ZT x LD Condition x Fluid Condition: n.s. (p= 0.3095)
	DS	n.s. (p= 0.1049)	**p= 0.0059**	**p< 0.0001**	ZT x LD Condition: n.s. (p= 0.1211) ZT x Fluid Condition: n.s. (p= 0.6872) LD Condition x Fluid Condition: n.s. (p= 0.3003) ZT x LD Condition x Fluid Condition: n.s. (p= 0.1332)
Clock	PFC	**p= 0.0418**	n.s. (p= 0.2656)	n.s. (p= 0.8571)	ZT x LD Condition: n.s. (p= 0.0774) ZT x Fluid Condition: n.s. (p= 0.1474) LD Condition x Fluid Condition: n.s. (p= 0.2531) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7353)
	NAc	n.s. (p= 0.7350)	n.s. (p= 0.8923)	n.s. (p= 0.3318)	ZT x LD Condition: n.s. (p= 0.4200) ZT x Fluid Condition: n.s. (p= 0.8517) LD Condition x Fluid Condition: n.s. (p= 0.7967) ZT x LD Condition x Fluid Condition: n.s. (p= 0.0632)
	DS	n.s. (p= 0.4188)	n.s. (p= 0.5801)	n.s. (p= 0.5889	ZT x LD Condition: n.s. (p= 0.1986) ZT x Fluid Condition: n.s. (p= 0.6346) LD Condition x Fluid Condition: n.s. (p= 0.1472) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7148)

ERβ	PFC	**p= 0.0415**	n.s. (p= 0.5835)	**p= 0.0437**	ZT x LD Condition: n.s. (p= 0.4458) ZT x Fluid Condition: n.s. (p= 0.7292) LD Condition x Fluid Condition: n.s. (p= 0.1893) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6341)
	NAc	n.s. (p= 0.1352)	n.s. (p= 0.2899)	n.s. (p= 0.2338)	ZT x LD Condition: n.s. (p= 0.8456) ZT x Fluid Condition: n.s. (p= 0.3247) LD Condition x Fluid Condition: n.s. (p= 0.5881) ZT x LD Condition x Fluid Condition: n.s. (p= 0.2671)
	DS	**p= 0.0030**	n.s. (p= 0.4058)	n.s. (p= 0.1459)	ZT x LD Condition: n.s. (p= 0.5813) ZT x Fluid Condition: n.s. (p= 0.1776) LD Condition x Fluid Condition: n.s. (p= 0.3637) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6530)
PR	PFC	n.s. (p= 0.6542)	**p= 0.0483**	n.s. (p= 0.6584)	ZT x LD Condition: n.s. (p= 0.7862) ZT x Fluid Condition: n.s. (p= 0.1362) LD Condition x Fluid Condition: n.s. (p= 0.3492) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6347)
	NAc	n.s. (p= 0.0798)	n.s. (p= 0.5399)	n.s. (p= 0.2874)	ZT x LD Condition: n.s. (p= 0.5353) ZT x Fluid Condition **p= 0.0459** LD Condition x Fluid Condition: n.s. (p= 0.9295) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6730)
	DS	n.s. (p= 0.3889)	n.s. (p= 0.5351)	n.s. (p= 0.1766)	ZT x LD Condition: n.s. (p= 0.9317) ZT x Fluid Condition: n.s. (p= 0.8058) LD Condition x Fluid Condition: n.s. (p= 0.4593) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6474)
mGluR5	PFC	n.s. (p= 0.4126)	n.s. (p= 0.9470)	n.s. (p= 0.1737)	ZT x LD Condition: n.s. (p= 0.6113) ZT x Fluid Condition: n.s. (p= 0.4981) LD Condition x Fluid Condition: n.s. (p= 0.6221) ZT x LD Condition x Fluid Condition: n.s. (p= 0.2912)
	NAc	n.s. (p= 0.5843)	n.s. (p= 0.2183)	n.s. (p= 0.0766)	ZT x LD Condition: n.s. (p= 0.2917) ZT x Fluid Condition **p= 0.0039** LD Condition x Fluid Condition **p= 0.0390** ZT x LD Condition x Fluid Condition: n.s. (p= 0.1359)
	DS	n.s. (p= 0.9071)	n.s. (p= 0.3266)	n.s. (p= 0.3422)	ZT x LD Condition: n.s. (p= 0.4925) ZT x Fluid Condition: n.s. (p= 0.6652) LD Condition x Fluid Condition **p= 0.0387** ZT x LD Condition x Fluid Condition: n.s. (p= 0.7295)

GluN2B	PFC	n.s. (p= 0.4958)	n.s. (p= 0.1562)	n.s. (p= 0.0610)	ZT x LD Condition: n.s. (p= 0.8064) ZT x Fluid Condition: n.s. (p= 0.3543) LD Condition x Fluid Condition: n.s. (p= 0.0713) ZT x LD Condition x Fluid Condition: n.s. (p= 0.8853)
	NAc	n.s. (p= 0.5085)	n.s. (p= 0.2876)	n.s. (p= 0.4104)	ZT x LD Condition: n.s. (p= 0.4687) ZT x Fluid Condition: n.s. (p= 0.2654) LD Condition x Fluid Condition: n.s. (p= 0.7785) ZT x LD Condition x Fluid Condition: n.s. (p= 0.5495)
	DS	n.s. (p= 0.5381)	n.s. (p= 0.4703)	n.s. (p= 0.3017)	ZT x LD Condition: n.s. (p= 0.8562) ZT x Fluid Condition: n.s. (p= 0.2523) LD Condition x Fluid Condition: n.s. (p= 0.0695) ZT x LD Condition x Fluid Condition: n.s. (p= 0.9955)
Kcnn2	PFC	**p= 0.0471**	n.s. (p= 0.7844)	n.s. (p= 0.3668)	ZT x LD Condition: n.s. (p= 0.7811) ZT x Fluid Condition: n.s. (p= 0.2633) LD Condition x Fluid Condition: n.s. (p= 0.7233) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6586)
	NAc	n.s. (p= 0.2198)	n.s. (p= 0.3944)	**p= 0.0352**	ZT x LD Condition: n.s. (p= 0.3744) ZT x Fluid Condition: n.s. (p= 0.8438) LD Condition x Fluid Condition: n.s. (p= 0.9572) ZT x LD Condition x Fluid Condition: n.s. (p= 0.8113)
	DS	n.s. (p= 0.1695)	n.s. (p= 0.3149)	n.s. (p= 0.8818)	ZT x LD Condition: n.s. (p= 0.7157) ZT x Fluid Condition: n.s. (p= 0.7579) LD Condition x Fluid Condition: n.s. (p= 0.7579) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7485)
TNFα	PFC	**p= 0.0040**	n.s. (p= 0.5850)	n.s. (p= 0.5784)	ZT x LD Condition: n.s. (p= 0.3050) ZT x Fluid Condition: n.s. (p= 0.6730) LD Condition x Fluid Condition: n.s. (p= 0.7818) ZT x LD Condition x Fluid Condition: n.s. (p= 0.0970)
	NAc	**p= 0.0421**	**p= 0.0158**	n.s. (p= 0.9826)	ZT x LD Condition: n.s. (p= 0.4758) ZT x Fluid Condition: n.s. (p= 0.2660) LD Condition x Fluid Condition: n.s. (p= 0.6879) ZT x LD Condition x Fluid Condition: n.s. (p= 0.6142)
	DS	**p= 0.0359**	**p= 0.0054**	n.s. (p= 0.2668)	ZT x LD Condition: n.s. (p= 0.6412) ZT x Fluid Condition: n.s. (p= 0.0643) LD Condition x Fluid Condition: n.s. (p= 0.2793) ZT x LD Condition x Fluid Condition: n.s. (p= 0.7507)

Table 6 Statistical analysis of gene expression of the core clock genes (Bmal1, Clock, Per2), sex hormone receptors (ERβ, PR), glutamate receptors (mGluR5, GluN2B), calcium-activated channel (Kcnn2), and pro-inflammatory marker (TNFα).

3.5 Discussion

The disruption of circadian rhythms caused by exposure to abnormal light-dark conditions is considered a risk factor for alcohol abuse (Barko et al., 2019). However, the neurobiological mechanisms underlying this relationship, particularly in females, are not well understood. In this study, we examined the relationship between circadian disruption and intermittent alcohol intake on the expression of genes in brain areas known to be involved in alcohol-drinking behavior. Consistent with previous findings, housing under LD22 conditions resulted in the desynchronization of locomotor activity rhythms, but it had no significant effect on alcohol consumption or preference (Meyer et al., 2022). The most pronounced effects of chronodisruption were observed in the expression levels of the *Bmal1* in both the dorsal and ventral striatum, whereas, the combination of chronodisruption and alcohol consumption had the greatest impact on the expression levels of the *TNFα*. However, expression levels of other clock genes and cell signaling genes remained mostly unchanged.

These results indicate that aberrant LD conditions and/or alcohol consumption only induce mild changes in molecular components within reward-related brain areas (overview Figure 22). This may explain why alcohol-drinking behavior remains similar under both standard and altered LD conditions in female rats. Based on the results, it can be concluded that chronodisruption does not have a significant influence on alcohol-drinking behavior in females.

	Gene	PFC	NAc	DS
Clock genes	***Bmal1***	ZT	ZT, ZT x LD	ZT, ZT x LD
	Per2	ZT, Fluid	ZT, Fluid, ZT x Fluid, ZT x LD	ZT, Fluid
	Clock	LD	-	-
Hormone receptors	***ERβ***	LD, ZT	-	LD
	PR	Fluid	ZT x Fluid	-
Glutamate circuit	***mGluR5***	-	ZT x LD, LD x Fluid	LD x Fluid
	GluN2B	-	-	-
	KCNN2	LD	ZT	-
Neuroinflammation	***TNFα***	LD	LD, Fluid	LD, Fluid

Figure 22 Summary of the effects of dysregulated circadian rhythms and alcohol consumption on the expression of circadian and cell-signaling genes in brain areas involved in the reward system in female rats.

3.5.1 Circadian rhythmicity and locomotor activity

Our study confirmed that LD22 exhibits chronodisruptive characteristics, consistent with previous findings in male rats (De La Iglesia et al., 2004). Interestingly, despite a significant decline in daily activity and consolidated rest-activity patterns observed in female rats, rhythm fragmentation within a day remained unchanged irrespective of the LD and fluid conditions. The utilization of non-parametric rhythmicity factors indicated that the reduction in rhythmicity stems from a lack of coordination between light and activity patterns across consecutive days, as evidenced by the decrease in interdaily stability (IS) and dampened robustness of activity (RA) in disturbed animals. However, no significant alterations were observed in intradaily variability (IV), suggesting that the disruption is primarily driven by impaired coordination over consecutive days rather than a more fragmented pattern within each day (Banks et al., 2022). Importantly, changes in the circadian output parameters have been reported in association with neurodegenerative diseases (Anderson et al., 2009; Hatfield et al., 2004).

Since previous research demonstrated that changes in circadian photoperiod and alcohol exposure can influence locomotor activity (Benstaali et al., 2002; Perreau-Lenz & Spanagel, 2015), we conducted a further assessment of home cage running wheel activity. Our findings revealed a significant decline in running wheel activity under LD22 conditions, while alcohol access did not affect the amount of wheel running. Prior studies have reported conflicting effects of non-standard LD conditions on locomotion. For example, male rats showed an increase in locomotion following repeated phase shifts (Okuliarova et al., 2016), whereas male mice exhibited a reduction in locomotion under short-term LL conditions (Bartoszewicz et al., 2009) as did exposure to dim light at night and a social jet lag protocol in male mice (Delorme et al., 2022). In female mice, both forward and inverted (shift work-like) photoperiod shifts were found to decrease locomotion (Banks et al., 2022). Furthermore, alcohol access in male mice has been reported to either promote locomotion or have no effect (Gamsby & Gulick, 2015; Smoothy & Berry, 1984). Notably, the reported changes in locomotor activity were not associated with any significant alterations in alcohol-drinking behavior under experimental LD conditions (Gamsby & Gulick, 2015; Rosenwasser et al., 2010). Hence, the employed experimental LD condition in this study may have a more pronounced impact on locomotor behavior compared to the used alcohol exposure paradigm, and the altered locomotor behavior does not appear to affect alcohol intake.

3.5.2 Alcohol drinking behavior

The used IAE20% paradigm involves daily cycles of intermittent alcohol drinking and abstinence, which mimics the repetitive pattern of excessive intake, abstinence, and relapse observed in individuals with alcohol abuse and dependence (Crabbe et al., 2011; Koob & Volkow, 2016). Previous research has demonstrated that the IA20% paradigm produces pharmacologically relevant blood alcohol concentrations in rats (Carnicella et al., 2014). Consistent with earlier studies using the IA20% paradigm in male rats (Mill et al., 2013; Simms et al., 2008), animals in our study exhibited increased alcohol consumption over time. However, we did not observe an effect of an aberrant light cycle on alcohol intake behavior, thus confirming the results of previous studies (Meyer et al., 2022; Rizk et al., 2022). Several factors such as strain, LD condition, and schedule of alcohol access may influence alcohol-drinking behavior and may contribute to these observed outcomes.

3.5.3 Gene expression

Non-standard LD conditions have been shown to disrupt the rhythmic expression of the core clock genes in various tissues (Oishi et al., 2015; Szántóová et al., 2011) and influence the diurnal regulation of reward-related processes (Depoy et al., 2017). Furthermore, alcohol exposure has been reported to alter molecular and behavioral rhythms (Guo et al., 2016) and affect the expression of genes associated with the reward system (Logan et al., 2014). Through gene expression analysis, we aimed to investigate the interplay of environmental circadian disruption and alcohol consumption on molecular targets involved in the circadian function and cell signaling in brain areas mediating reward processes.

Gene association studies in humans have linked the core clock genes *Bmal1*, *Per2*, and *Clock* to alcohol consumption (Dong et al., 2011; Kovanen et al., 2010), and animal studies have supported these findings. Mutations of *Per1*, *Per2*, and *Clock* in mice have been shown to enhance alcohol consumption, likely through alterations in reward processes (Gamsby et al., 2013; Ozburn et al., 2013; Rizk et al., 2022; Spanagel, Pendyala, et al., 2005), whereas *Cry1/2*-deficient mice exhibited decreased alcohol preference (Hühne et al., 2022). Notably, a targeted knockout of *Bmal1* specifically in medium spiny neurons (MSNs) of the striatum decreased alcohol consumption in female mice (de Zavalia et al., 2021), while *Bmal1* ablation in the NAc resulted in higher alcohol consumption (Herrera et al., 2023). Moreover, the selective deletion of

Per2 in either the entire striatum or the NAc did not affect alcohol consumption in female mice (de Zavalia et al., 2021; Herrera et al., 2023). The results suggest that clock genes may have inhibitory or stimulatory effects on alcohol consumption in females, depending on the specific clock gene and brain region, highlighting the intricate regulation of alcohol consumption by these genes.

Our data indicated differential effects of aberrant light conditions and alcohol consumption on the mRNA expression of the clock genes *Clock*, *Bmal1*, and *Per2* in female rats. Interestingly, *Clock* expression remained unaffected by photoperiod and alcohol exposure, which may contribute to the observed stability in alcohol consumption levels in female rats. Previous findings by Ozburn et al. (2013) showed that mice bearing a *Clock* gene mutation exhibited significantly increased alcohol intake, particularly in females. Moreover, further investigation revealed that both female WT and C*lock*-deficient female mice consumed similar amounts of alcohol over time unlike the observed differences in drinking behavior within male cohorts (Rizk et al., 2022). Notably, these effects were not influenced by the alterations in the LD schedule (Rizk et al., 2022).

In the present study, the expression of *Bmal1* in the striatum and prefrontal cortex was influenced by environmental light conditions. However, the changes in *Bmal1* expression associated with LD22 did not correspond to the unaffected alcohol intake. Thus, the observed daily fluctuations in *Bmal1* expression in the striatum may have a relatively weak impact on alcohol consumption compared to a complete gene knockout, as demonstrated in mice (de Zavalia et al., 2021; Herrera et al., 2023). *Bmal1* in the striatum may have a direct effect on alcohol consumption rather than a rhythmic regulation of clock-controlled processes influencing alcohol drinking in females. Alternatively, it can be argued that certain clock-controlled processes may remain unaffected by the *Bmal1* dysregulation, as indicated by the levels of *Per2* expression in the mPFC, NAc, and DS under LD22 conditions, potentially mitigating the effect of chronodisruption on alcohol consumption. Although the persistent differences in *Per2* expression at ZT2 and ZT14 under LD22 conditions were unexpected, they align with trends observed in other studies. For example, male and female mice with a striatum-specific deletion of *Bmal1* showed mostly unaltered *Per2* expression in the dorsal striatum (Schoettner et al., 2022). Additionally, male mice exposed to aberrant LD conditions display disrupted *Per2* expression in the SCN, while robust expression

profiles of *Per2* were observed in the striatum (Ikeno & Yan, 2016). These findings suggest that other factors, such as dopamine signaling, may influence the expression of clock genes in the mesolimbic system, as demonstrated by Hood et al. (2010). Despite recent advances, further studies are needed to better understand the interaction of the cell signaling pathways, such as the dopaminergic circuit, the circadian clock, or individual clock genes in the reward system, and how they influence drug-related behaviors.

Although the expression of *Per2* in the NAc is moderately reduced in female rats during acute alcohol exposure, it does not appear to affect drinking behavior itself. Previous studies have associated *Per2* expression with alcohol consumption in rodents, but recent findings in mice suggest that these effects are sex-dependent. For example, the global or striatum-specific knockout of *Per2* in male mice increased alcohol consumption (de Zavalia et al., 2021; Herrera et al., 2023; Spanagel, Pendyala, et al., 2005). In contrast, female mice with striatum-specific *Per2* knockout display no change in alcohol consumption, indicating that other sex-specific factors must be considered in the control of alcohol-drinking behavior in females.

A potential link between clock genes, sex, and brain region in relation to alcohol consumption in females may involve the signaling of sex hormones. Previous studies have shown that CLOCK-BMAL1 complexes bind to the E-box element in the promoter region of estrogen receptor β (ERβ), thereby influencing its expression and subsequently altering cell signaling pathways, such as glutamatergic signaling in the striatum of female rats (Grove-Strawser et al., 2010), which may indirectly affect alcohol consumption (Pandey et al., 2004). In our study, we observed that the levels of ERβ mRNA remained consistent regardless of LD conditions, variations in Bmal1 expression, or alcohol consumption. To investigate whether estrogen signaling is influenced by exposure to abnormal LD conditions and/or alcohol consumption, future research should examine the dynamics of sex hormone fluctuations in animals kept under chronodisruptive LD schedules.

It is widely recognized that changes in the glutamate circuit have an impact on reward-related behaviors, including alcohol consumption, and vice versa (Chandrasekar, 2013; Eisenhardt et al., 2015; Gass & Olive, 2008). Numerous studies have established a connection between the metabotropic glutamate receptor subtype-5 (*mGluR5*) and the regulation of various behavioral pathologies associated with alcohol misuse. Previous research has demonstrated that alcohol

consumption modulates the *mGluR5* in a brain-region-specific manner (Cozzoli et al., 2009; Simonyi et al., 2004) and that *mGluR5* receptor manipulations affect alcohol intake (Cozzoli et al., 2014; Goodwani et al., 2017). In our study, we found a significant interaction of LD22 and IA20% on *mGluR5* mRNA levels in the NAc and DS. Furthermore, the inotropic NMDA receptor serves as a key target for the actions of alcohol within the central nervous system, and behavioral and cellular investigations have pointed to the importance of the *GluN2B* subunit in mediating the effects of alcohol (Allgaier, 2002; Nagy et al., 2005). NMDA receptors are implicated in synaptic plasticity and are involved in various aspects of drug and alcohol addiction (Abrahao et al., 2013; Kroener et al., 2012). However, the effects of alcohol on NMDA are inconsistent, as acute alcohol exposure has been shown to inhibit, while chronic exposure promotes the function of the *GluN2B* subunit (Nagy et al., 2003; Sheela Rani & Ticku, 2006; Wang et al., 2007). Interestingly, our data did not reveal any changes in *GluN2B* mRNA expression following chronic intermittent alcohol access.

Additionally, small-conductance calcium-activated potassium channels (SKs) modulate NMDA receptor-dependent synaptic plasticity (Faber et al., 2005; Lin et al., 2008) and facilitate learning and memory acquisition (Hammond et al., 2006; Sun et al., 2020). Chronic alcohol exposure in mice led to a reduction in the expression of *Kcnn2* (KCa2.2) and a significant increase in the expression level of *GluN2B* (Mulholland et al., 2011). In contrast, we reported LD-dependent changes in *Kcnn2* in the PFC but did not observe any associated effects of alcohol consumption. Taken together, previous research has linked alcohol consumption with alterations in the glutamate circuit and vice versa, but our data revealed only moderate changes in the expression of *mGluR5*, *GluN2B*, and *Kcnn2*, which aligns with the observed lack of changes in alcohol consumption.

In addition to their influence on cell signaling, a growing body of evidence supports the pivotal role of the circadian clock genes in the regulation of inflammatory processes (Carter et al., 2015), which potentially contribute to alterations in alcohol consumption. Studies have shown that the deletion of *Bmal1* and *Rev-erbα* leads to widespread glial activation, inflammation, and oxidative stress in the brain (Griffin et al., 2019; Musiek et al., 2013). Likewise, chronic alcohol intake has been reported to induce neuroinflammation characterized by increased expression of proinflammatory cytokines, such as tumor necrosis factor-alpha (*TNFα*) (Blednov et al., 2005,

2011; Cooper et al., 2020; Kelley & Dantzer, 2011). Activation of *TNF*α has been associated with impaired neuronal functioning and increased risk of alcohol abuse and relapse (Obad et al., 2018). Our findings demonstrate that chronic alcohol exposure, as well as LD22 cycles, independently amplify *TNFα* mRNA levels in all examined brain regions. However, *TNFα* is a nonspecific inflammatory marker, and future studies should investigate the complex relationship between circadian disruption, clock genes, and the neuroinflammatory processes.

Both, this study and previous work in mice indicate that female rodents may exhibit greater resilience to aberrant light conditions concerning alcohol consumption. However, a confounding factor in these findings may be the use of running wheels. Exercise is known to influence alcohol-drinking behavior (Buhr et al., 2021; Gallego et al., 2015; Werme et al., 2002). Consequently, it may be intriguing for future studies to compare the effect of LD changes on alcohol intake and gene expression in the presence and absence of running wheels.

3.6 Conclusion

The results of this study show that environmentally-induced circadian rhythm disruptions did not affect alcohol-drinking consumption in females despite dysregulation of *Bmal1* expression in several brain regions associated with alcohol-drinking behavior. It is possible that other clock genes associated with alcohol consumption, like *Per2,* maintain rhythmicity in brain areas important for reward processing, or do not change their expression profile, like *Clock* may contribute to compensatory mechanisms to counteract the loss of *Bmal1* rhythmicity. Our data indicated a more intricate pattern of clock gene expression in the striatum, a brain region that governs alcohol drinking in a sex-dependent manner in rodents, regardless of the presence or absence of specific clock genes. Future studies should focus on specific neural circuits to elucidate relations between molecular changes and behavioral phenotypes in females. In particular, further research is needed to investigate pathways that are typically associated with alcohol consumption but were mostly unaffected in the female rats examined in this study.

General discussion

Chronodisruption affects mental health, such as mood and anxiety or substance abuse, however, most pre-clinical and clinical research addressed important questions on phenology and underlying mechanisms in male subjects only. Thus, the validity of those outcomes for females remains unclear. The aim of this thesis was to examine the long-term effects of circadian desynchronization caused by exposure to aberrant light-dark (LD) cycles on physiology and behavior as well as brain-related processes in female rats.

The findings of this thesis indicate that chronodisruption induces physiological changes such as irregular locomotor activity rhythms, reduced wheel-running activity, and estrus cycle instability in female rats accompanied by a loss of daily differences in *Bmal1* and reduced *Per2* expression in fore- and midbrain areas, but did not affect mood-related performance and alcohol consumption (Figure 23). In contrast, behavioral changes, including anxiety-like behavior were observed during alcohol abstinence. These behavioral changes were only marginally related to the degree of circadian disruption, suggesting that other external and/or internal factors are involved. A common shared feature of chronodisruption and abusive alcohol consumption is the induction of inflammatory processes in brain regions associated with the reward system, which is consistent with the results of this study.

The outcomes of this research project provide compelling evidence that environmentally-induced chronodisruption in female rats is a risk factor for the development of mood disorders in combination with other stress factors like alcohol abstinence.

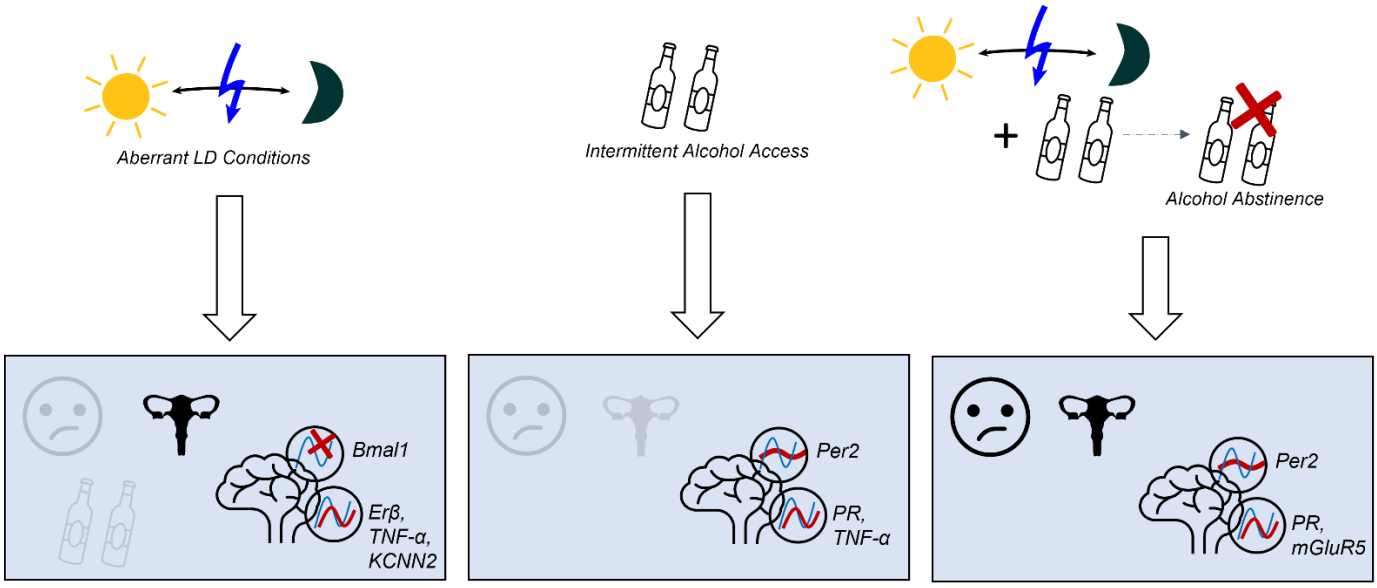


Figure 23 Graphical summary of the effects of experimental LD conditions (LD22, SHIFT) on alcohol intake behavior, behavioral performance, and molecular consequences in brain regions involved in the reward circuit in female rats.

Consequences of chronodisruption on mood-related behavior and alcohol consumption in female rats

The prevalence of long-term circadian disruption caused by abnormal light exposure is increasing in our society. In this study, we aimed to simulate internal desynchrony using repeated phase shifts and a short-day paradigm, representing two common forms of aberrant light schedules in modern society. Both paradigms presented a chronic re-entrainment challenge, as the organism could not fully adapt to the new LD condition before the next shift occurred (Roenneberg et al., 2003). Similar to the findings of Banks et al. (2022), the experimental LD schedules used in our study led to decreased activity due to poor synchronization of activity rhythms with the external LD cycles across consecutive days. Additionally, we observed disruptions in the estrus cycle, further confirming the impact of chronodisruption on physiology. Surprisingly, despite these disturbances, we did not observe significant changes in alcohol consumption and mood-related behavior in female rats exposed to aberrant LD conditions.

In contrast, our study revealed alterations in the expression of the core clock genes *Bmal1*, *Per2*, and *Clock* in the prefrontal cortex (PFC), nucleus accumbens (NAc), and dorsal striatum (DS). Previous research has demonstrated that changes in clock gene expression in the forebrain and

midbrain play a crucial role in the development of psychopathological conditions (Logan & McClung, 2018; McClung, 2007). Specifically, the contribution of *Bmal1* and *Per2* expression in neurons and glial cells has been receiving considerable attention over the past years (Becker-Krail et al., 2016). Our findings showed that the combination of abnormal light/dark cycles and alcohol exposure had differential effects on clock gene expression across brain regions, with relatively similar outcomes. Interestingly, no significant differences in expression levels of *Bmal1* between ZT2 and ZT14 were found in rats exposed to aberrant LD conditions when compared to controls. On the other hand, *Per2* expression showed diurnal variation, albeit slightly reduced in amplitude.

Noteworthy research in male and female mice with conditional ablation of *Bmal1* or *Per2* in the striatum reported only mild changes in affective behaviors (Schoettner et al., 2022), but had profound sex-specific effects on alcohol consumption (de Zavalia et al., 2021). Studies on genome-wide *Bmal1* knockout mice indicate an antidepressant effect of the knockout (Leliavski et al., 2014), whereas *Bmal1* knockdown in the SCN accompanied by a suppression of *Per2* expression in mood-regulating brain areas significantly induced depressive- and anxiety-like behavior (Landgraf et al., 2016). Collectively, these results suggest that the effects of clock gene expression on mood regulation might be dose-dependent (Porcu et al., 2020). It is essential to acknowledge the importance of distinguishing between different types of clock gene manipulations (knockout vs. knockdown vs. dysregulation) and their specific effects on the regulation of affective behaviors. The discrepancies in the literature underscore the complexity of these interactions and call for further research to gain a comprehensive understanding of the mechanisms involved.

Similarly, when considering the recently identified role of striatal-specific *Bmal1* and *Per2* expression in the sex-dependent regulation of alcohol consumption in mice (de Zavalia et al., 2021; Herrera et al., 2023) we hypothesized that changes in *Bmal1* and *Per2* expression in the striatum of chronodisrupted rats may be indicative of altered alcohol consumption. However, this study demonstrated that despite their dysregulation, alcohol drinking remained unaffected in female rats. The extent to which the manipulation of one clock gene affects the function of the other is uncertain. While the loss of *Bmal1* appears to influence *Per2* expression, the profiles of *Per2* expression in mice with a conditional knockout of Bmal1 are remarkably similar to control

animals (Schoettner et al., 2022). This suggests that other factors, such as dopamine signaling as proposed by Hood et al. (2010), may contribute to *Per2* expression independently of local *Bmal1* abundance.

The intricate relation between *Bmal1* and *Per2* expression and their role in alcohol consumption may be also sex-dependent, and exclusive to particular brain regions. For example, the deletion of *Bmal1* in the entire striatum increases the propensity to consume alcohol in male mice but decreases it in females (de Zavalia et al., 2021). Similarly, *Per2* deletion from the entire striatum increased alcohol consumption in males but had no effect in females. This implies a shared mechanism of *Bmal1* and *Per2* in regulating alcohol consumption in males, but not in females. Furthermore, NAc-specific deletion of *Bmal1* in female mice reversed the alcohol-drinking phenotypes observed in animals with a deletion of *Bmal1* in the entire striatum, indicating that clock genes in subregions of the striatum contribute differently to the propensity to drink alcohol in females (Herrera et al., 2023). Thus, the combined response of each respective brain area, in which clock gene expression is affected through chronodisruption, might attenuate the development of robust drinking phenotypes in females.

Indeed, clock gene manipulations have been linked to alcohol-drinking phenotypes in previous studies (Dong et al., 2011; Spanagel, Pendyala, et al., 2005). However, attempts to replicate these findings through environmental manipulations have not been successful. The key difference lies in the specificity of genetic manipulations, which target particular components of the circadian system, leading to distinct outcomes compared to environmental approaches that affect various neurophysiological pathways. For example, a study by Rizk et al. (2022) revealed that environmentally-induced circadian disruption had no impact on alcohol intake in female mice, whereas a targeted manipulation of clock gene expression altered drinking behavior in females in the same study. These findings strongly suggest that clock genes play a significant role in regulating alcohol consumption, but their effects are most notable when the genes are absent, rather than when they are dysregulated. Future research should prioritize direct comparisons between genetic and environmentally-induced circadian disruptions, while also implementing tailored experimental designs that account for sex as a crucial factor. This approach will contribute to a more profound comprehension of how the circadian system impacts the development of psychopathological conditions, including alcohol-related disorders.

Importantly, downstream processes, such as cell signaling, which may be disturbed by chronodisruption, showed only minor effects. We observed that neither the LD conditions nor alcohol access strongly impacted glutamate signaling (*mGluR5*, *GluN2B*, *KCNN2*) and sex hormone expression (*ERβ*, *PR*). Previous research has shown that the clock gene *Per2* influences the glutamatergic system, subsequently modulating alcohol consumption (Spanagel, Pendyala, et al., 2005). Similarly, clock gene components can influence hormone receptors, such as Erβ (Cai et al., 2008). These findings may provide an explanation for the behavioral stability observed despite chronodisruption.

Furthermore, it is crucial to consider other factors that may mitigate the effects of chronodisruption on mood and alcohol consumption (Figure 24). In our study, running wheels were consistently utilized to monitor locomotor activity. Previous research has indicated that exercise, in the form of running wheel usage, can influence dopamine signaling in the striatum (Bastioli et al., 2022). Interestingly, exercise has been shown to offer protection against circadian disruptions (Leise et al., 2013). It aids by entraining the body to external rhythms and enhances the internal synchronization of various circadian rhythms (reviewed by Weinert & Gubin, 2022). Notably, running wheel-based exercise has demonstrated promising results in attenuating stress-induced mood changes (Lee et al., 2015; Tal-Krivisky et al., 2015) and it has shown efficacy in countering behavioral alterations associated with alcohol abstinence in male mice (Sampedro-Piquero et al., 2020). Given these findings, it would be intriguing to compare the experiments conducted in Chapters 1, 2, and 3, to experiments where no wheels had been used. Such a comparison could provide valuable insights into the role of exercise and its potential influence on mood-related behavior and alcohol consumption in chronodisrupted animals.

Additionally, genetic variability in circadian phenotypes, such as shorter wheel-running periods, has been linked to high-alcohol preferring mice (McCulley et al., 2013). Selectively bred rat lines with distinct behavioral phenotypes associated with alcohol use disorders have also been identified (Lundberg et al., 2022). For instance, HAD2 rats display a behavioral profile resembling individuals with higher negative emotionality, which is linked to a vulnerability to developing alcohol use disorders. Understanding the relationship between individual circadian parameters, emotionality indices, and future alcohol intake could offer valuable insights into the complex interactions between circadian rhythms, behavior, and alcohol consumption.

The majority of previous research has been conducted using male subjects. For instance, male rats exposed to LD22 (Ben-Hamo et al., 2016), repeated phase shifts (Horsey et al., 2020), or dim light (Fonken et al., 2012) display anxiety-like and depressive-like behavior. These findings suggest that chronic circadian disruption impacts the function of neural circuits important for mood regulation (Logan & McClung, 2018), but its implications for the female organism remain unclear. Our study reports, for the first time, that a repeated jet lag paradigm and short light-dark schedules did not induce mood-related changes in female rats. These results suggest that sex differences may play a role in our observations. Indeed, previous transcriptome analysis by Paden et al. (2020) highlighted gonadal and genetic sex differences in mood-relevant brain regions, providing molecular insights into sex-based behavioral variations. Specifically, stress was found to produce sex-specific changes in mesocorticolimbic circuitry, showing distinct molecular profiles in the brains of males and females exposed to chronic stress (Paden et al., 2020).

Circadian disruption and alcohol are recognized as stress factors that can significantly impact health (Sarkar, 2012). The interaction between the circadian system and stress responses has been found to influence mood regulation (Landgraf et al., 2014) and alcohol consumption (Peltier et al., 2019). Mutations in *Clock*, *Cry*, and *Per* genes have also been associated with changes in glucocorticoid levels and rhythmicity (Becker-Krail et al., 2016; Koch et al., 2017). For example, *Bmal1* mutant mice exhibit decreased glucocorticoid levels, linked to reduced stress-induced glucocorticoid levels and depressive-like behavior in the forced swim test (Leliavski et al., 2014). Additionally, long-term alcohol consumption and abstinence disrupt not only the HPA axis but also activate extrahypothalamic brain stress systems, which play a complex and dynamic role in influencing drinking behavior, independent of neuroendocrine mechanisms (Koob & Kreek, 2007). The findings of this thesis indicate that female subjects exhibit behavioral changes when exposed to a combination of chronodisruption and alcohol abstinence stress, while previous research suggested that mood-related changes in males were induced by chronodisruption alone. This suggests that sensitivity to potential stressors may vary between the sexes, as previous studies have reported sex differences in stress responses and regulation, possibly related to variations in neural responses to acute stressors between men and women (Goldfarb et al., 2019). To gain a comprehensive understanding of the interplay between circadian disruption and alcohol consumption, along with subjective experiences, future studies

could explore corticosterone levels and other stress-related markers, as well as potential changes in the adrenal gland during alcohol abstinence under both standard and aberrant LD conditions. Further research should explicitly examine sex-specific neural responses during different stress factors such as circadian disruption and alcohol to better understand stress regulation and output behavior for each sex.

Another intriguing finding of this study is that both circadian disruption and alcohol consumption independently trigger inflammatory responses in specific brain regions associated with the reward system. Both circadian disruption and alcohol have been shown to affect inflammatory processes in the body. As circadian disorganization in male mice leads to disruption of immune and inflammatory responses (Castanon-Cervantes et al., 2010; Summa et al., 2013) alcohol affects innate immune signaling in different brain cell types by altering gene expression and molecular pathways responsible for regulating neuroinflammation (Erickson et al., 2019). As a result, alcohol-induced systemic inflammation impairs neuronal function and may contribute to alcohol withdrawal symptoms such as autonomic disturbances and anxiety (Retson et al., 2015). Females may be more sensitive to alcohol-induced neuroimmune responses (Baxter-Potter et al., 2017). Consequently, the process of neuroinflammation induced by circadian disruption and alcohol plays a crucial role in alcohol abuse behavior and affects mood performance, but their combinatory effect on females is still poorly understood. The relationship between circadian disruption and its influence on inflammatory processes, as well as its interaction with alcohol-induced inflammation, is undoubtedly complex and likely influenced by various other factors. To fully comprehend this intricate relationship, additional research is imperative.

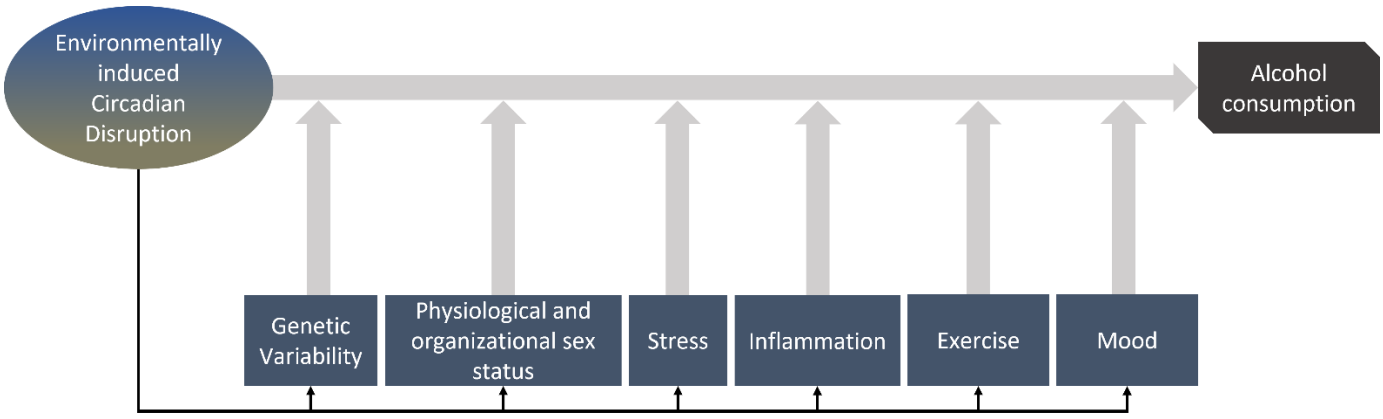


Figure 24 Factors influencing alcohol consumption in female rats.

Are females more vulnerable to the effects of chronodisruption?

The circadian timing system regulates the female sex hormone system, while sex hormones, in turn, affect the circadian timing system. Sex hormones act throughout the entire brain via genomic and non-genomic receptors, and altered sex hormones levels are impacting numerous neural, physiological, and behavioral functions, such as mood disorders and neuroinflammatory processes often in a sex-dependent manner (Ho et al., 2019; Li & Graham, 2017; Marrocco & McEwen, 2016; McEwen & Milner, 2017; Siddiqui et al., 2016). For example, higher estrogen levels have been associated with increased alcohol consumption in females (Erol et al., 2019). This association is linked to the interaction between estrogen and progesterone with neurotransmitters, particularly dopamine, which plays a significant role in mediating the effects of alcohol (Barth et al., 2015).

Our findings indicate that circadian disruption affects estrus stability and regularity, but only minor effects are observed on sex hormone receptor gene expression, such as estrogen receptor beta (Erβ) and progesterone receptor (PR) in different brain tissues. The LD-induced peripheral sex hormone impairment did not alter subsequent mood-related behavior and alcohol consumption. This finding is intriguing as the female organism poses a circadian clock in the ovaries, known as the ovarian clock (Fahrenkrug et al., 2006; Sellix, 2015), that has not been yet identified in male reproductive tissues (Morse et al., 2003). The ovarian clock is SCN-dependent and highly influenced by sex hormones, playing a crucial role in sex hormone production (Yoshikawa et al., 2009). Additionally, genes associated with steroidogeneses, like the steroidogenic acute regulatory protein (StAR), and other sex hormone-converting enzymes (e.g.,

Lhcgr, CYP17A1) exhibit rhythmic transcript profiles in ovarian tissue and are affected by circadian manipulation in peripheral (Chen et al., 2013; Wang et al., 2020) and central tissues (Chustecka et al., 2020) suggesting their direct regulation by the circadian timing system. Thus, a disruption of the ovarian clock, but also sex hormone-converting enzyme systems may have consequences for sex hormone levels, and subsequently for sex hormone-related processes and pathologies, impacting health. Our findings require more research on the action of peripheral sex hormones and central-related effects. It would be intriguing to explore potential changes in hormone levels in both peripheral and central tissues, along with corresponding sex hormone receptors, after chronic and acute environmental disruption and their implications on tissue-specific central processes like neurogenesis and neuroprotection.

Conclusion

This thesis represents a pioneering exploration into the effects of chronodisruption and alcohol consumption on the female organism. The results indicate that chronodisruption may act as a stress factor, which, when combined with other stressors like alcohol abstinence, increases the risk of developing affective disorders, such as anxiety. While chronodisruption affects various physiological and molecular processes in the fore- and midbrain, none of these factors can be considered the sole cause of behavioral alterations. Further studies may investigate the effect of that exercise in the form of running wheels as a possible tool in mitigating the consequences of chronodisruption on females.

Our findings emphasize the importance of circadian hygiene for women working under long-term shiftwork conditions. Understanding the potential adverse effects of chronodisruption and alcohol on females can aid in optimizing circadian rhythms and reducing the detrimental consequences associated with shiftwork.

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